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Determining TrkB intracellular signalling pathways required for specific aspects of gustatory development

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Thesis submitted to the School of Biomedical Sciences, College of Medicine and Veterinary Medicine at the University of Edinburgh for the degree of Doctor of Philosophy
2013

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ABSTRACT

Neurotrophins BDNF and NT4 influence the development of the rodent gustatory system. Despite binding to the same receptor, TrkB, they have different roles. BDNF is chemo-attractive for gustatory neurons and regulates gustatory neuron targeting and number during development. NT4 regulates gustatory neuron number earlier in development than BDNF, but it is not chemo-attractive and does not regulate gustatory neuron targeting. To elucidate the mechanisms that regulate these processes we have examined which TrkB intracellular signalling pathways are required for specific aspects of gustatory development by studying the effect of specific point mutations in TrkB docking sites. We found that the TrkB/Shc docking site is involved in regulating the survival of geniculate ganglion neurons as a point mutation in this adaptor site (*Trkb*$^{S/S}$) caused large losses of these neurons as early as E12.5. These losses were exacerbated throughout development until after birth. A point mutation in the TrkB/PLCγ (*Trkb*$^{P/P}$) docking site did not cause loss of geniculate ganglion neurons at any point during development. Animals with a point mutation in both docking sites (*Trkb*$^{D/D}$) caused a further decrease in neuron numbers compared to animals with a mutation in only one of the docking sites, similarly to what has previously been shown in *Trkb* null animals. We concluded that the TrkB/Shc docking site is crucial for determining the survival of geniculate ganglion neurons during mouse gustatory development, while the TrkB/PLCγ docking site does not affect the neuronal survival directly and likely plays a role in maintenance of these neurons.

Examining the targeting of geniculate ganglion afferents into the tongue revealed large deficits in innervated neural bud and taste bud numbers in *Trkb*$^{S/S}$ animals both before and after birth. This was concluded to be reflecting the lack of neuronal survival in this ganglion, a result that was mirrored in *Trkb*$^{D/D}$ animals. *Trkb*$^{P/P}$ animals, on the other hand, exhibited a developmental delay in innervation. This was indicated by a low amount of innervated neural buds following the initial innervation period, which was compensated for by a large increase in the number

of innervated taste buds by birth. By adulthood, the numbers of taste buds present on the tongues of *Trkb*$^{P/P}$ animals reached normal numbers compared to control animals. This suggested that the TrkB/PLCγ docking site is involved primarily in innervation.

Finally, we examined the morphology of taste buds in newly born and adult animals. We found that the low amount of geniculate ganglion afferents innervating the tongue in *Trkb*$^{S/S}$ and *Trkb*$^{D/D}$ animals caused a decrease in size of taste buds. This effect was seen to be partially rescued by adulthood in *Trkb*$^{S/S}$ animals but not in *Trkb*$^{D/D}$ animals due to lack of viability. The morphology of taste buds was unaffected in *Trkb*$^{P/P}$ animals until adulthood, at which point the size of the taste buds was increased. These results are in agreement with previous findings showing dependency of taste bud morphology on the amount of innervation.

Overall, our findings show a differential role of TrkB adaptor sites in gustatory development. Despite activated by the same ligands, the docking sites on this receptor are able to exert different influence on signalling pathways downstream of TrkB affecting neuronal survival, targeting and morphology of taste buds.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my PhD supervisor, Dr. Liliana Minichiello for the guidance and advice throughout my PhD. Her help was invaluable, particularly during the last months of this project.

I would also like to thank all the former and present members of the Minichiello lab, especially Dr. Mirjam Geibel and Dr. Dario Besusso for their encouragement, support, and help through my PhD, as well as their patience while teaching me all the methods I would be using during my project. I am also grateful to Dr. Jacqui Horn for her assistance with the maintenance and genotyping of the mouse lines. All the members of the lab created a nice working environment and it was a pleasure to work with them for the past three years. Furthermore, I would like to thank Professor Peter Brophy and the Centre for Neuroregeneration for their financial support particularly during the last six months, which enabled me to finish my project.

There are also many people who I am thankful to for their support outside of the university, and I will try to thank them in person. Particularly my family, who often offered emotional support and encouragement that made this project and the write up of the thesis possible, as well as my friends in Edinburgh who made sure I would not spend all my free time working in the labs.

Finally, I would like to thank my partner, Sigrid, who has provided me with encouragement, love and support during the PhD but most importantly the write up as the project came to an end.

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1. INTRODUCTION

1.1 Neurotrophins and their receptors

1.1.1 Structure of neurotrophins

Neurotrophins are a small family of growth factors that can influence cell dynamics, guidance, survival and development of sensory and sympathetic neurons. Their existence came into view for the first time in the 1950s when Rita Levi-Montalcini, Stanley Cohen and Viktor Hamburger discovered the existence of NGF, the nerve growth factor (Cohen et al. 1954), a substance that was found to be influencing tissue innervation in the peripheral nervous system. This discovery was followed by isolation of the brain-derived neurotrophic factor, BDNF (Hofer and Barde 1988), from pig brain. Following the identification of the rest of the neurotrophin family, neurotrophins have been found to be expressed in most neurons, where they influence neuronal survival, development, as well as shape and guidance (Huang and Reichardt 2001, Reichardt 2006).

Altogether there are four neurotrophins in mammals. Aforementioned NGF and BDNF are complemented by neurotrophin 3 (NT3) and neurotrophin 4/5 (NT4/5). There are also two other neurotrophins, NT6 and NT7, that have been found in fish (Gotz et al. 1994, Huang and Reichardt 2001) and will not be discussed in this thesis.

Developmentally, neurotrophins are important in terms of target innervation. As they are expressed in target regions of sensory axons, they are thought to provide trophic support and guidance cues to neurons that have not contacted their final targets yet (Farinas et al. 1996, Huang et al. 1999, Ringstedt et al. 1999), as has been shown for instance with BDNF and NT4 and their influence on geniculate ganglion fibers into the tongue during the rodent gustatory development (Ringstedt et al. 1999, Krimm et al. 2001). Their influence can be facilitated either extrinsically or intrinsically (Huang et al. 1999).

All of the neurotrophins are expressed throughout both the peripheral nervous system as well as the central nervous system and the levels vary throughout development. The highest levels of BDNF, NGF and NT3 mRNA have

been found in the adult mouse hippocampus (Hofer et al. 1990, Dawbarn and Allen 2003).

1.1.2. Neurotrophin receptors

Generally, neurotrophins function as non-covalently associated homodimers, although there are some subunits that can form heterodimers as well (Huang and Reichardt 2001). Their sequences are highly conserved in mammals (with the exception of NT4). In order to exert influence on a cell, neurotrophins must first be internalized by a receptor-dependent mechanism, and subsequently transported (along axons for instance) in vesicles towards their target area of action (Huang and Reichardt 01).

Neurotrophins bind to two different classes of transmembrane receptor proteins: receptor tyrosine kinases (Trk) and the neurotrophin receptor p75 (p75NTR) (Bibel and Barde 2000). Each of these is able to activate an explicit receptor kinase, although all of them (in their pro-forms) are able to activate the p75NTR (discussed below). Specifically, NGF activates TrkA, BDNF and NT4 preferentially activate TrkB, and NT3 activates TrkC; although NT3 is also able to activate TrkA and TrkB (Barbacid 1995, Bibel and Barde 2000), (Figure 1). Activation of the neurotrophin receptors can lead to opposite actions particularly in matters concerning cell death.

All neurotrophins are initially synthesized as precursors (proneurotrophins), which dimerize after translation (Kolbeck et al. 1994), and are proteolytically cleaved to mature neurotrophins. Proneurotrophins can be secreted from cells, or they are cleaved by furin or pro-convertases to form C-terminal mature neurotrophins (Teng et al. 2005, Lee et al. 2001a). Proneurotrophins that aren't cleaved signal primarily by binding and activating p75NTR (Lu et al. 2005).

The prodomains of these proneurotrophins show large sequence homology, are conserved among vertebrates (Lu et al. 2005), and have been shown to promote folding of the mature domains of neurotrophins (Suter et al. 1991, Rattenholl et al.

2001). These prodomains are also important for intracellular trafficking and secretion of neurotrophins (Lu 2003).

Because it is the proneurotrophins that signal through p75NTR to exclusively induce apoptosis (since neurotrophins can do this either by binding either the Trk receptors or the p75NTR receptor), the widespread expression of p75NTR itself is not sufficient to determine which of the apoptotic or survival actions of cells will predominate; it is rather the expression and secretion of the pro-forms of neurotrophins (Lee et al. 2001a) that ultimately determines the fate of the cell.

p75NTR

P75NTR is a member of the tumour necrosis factor receptor family (Chao 1994) and the FAS/Apo-1/CD95 family (Bibel et al. 1999), with molecular weight of 75 kDa (Kenchappa et al. 2006). The family of these receptors is defined by ligand-binding domains consisting of one or more repeats of a 40 amino acid cystein-rich domain (Schecterson and Bothwell 2010, Johnson et al. 1986).

Activation of p75NTR mediates cell death in sympathetic ganglia (Bamji et al. 1998) in a ligand-dependent fashion and it has been found to play a role in cell death in retina, spinal cord as well as basal forebrain (Frade and Barde 1998, 1999, Naumann et al. 2002). Furthermore, p75NTR induces apoptosis in the CNS following spinal cord lesion (Beattie et al. 2002).

Many neuronal populations co-express Trk receptors as well as p75NTR receptor, which suggests the possibility of an interaction between the two receptors deciding the faith of a particular cell. Bibel et al. (1999) showed that p75NTR coimmunoprecipitated with other Trk receptors, which increased the responsiveness of transfected cells to low neurotrophin concentrations. The interactions between the two types of neurotrophin receptors seem to be mediated by both the extracellular and intracellular domains as either one seems to be sufficient to drive a stable association of these receptors. However, in the absence

of receptor phosphorylation, it is the extracellular domain that drives this interaction (Bibel et al. 1999).

One of the requirements for binding of neurotrophins to the p75NTR receptor and subsequent intracellular signaling is the dimerization of the receptor. This occurs by formation of a disulfide bridge produced via cysteine residues within the transmembrane domains, which is then separated into two intracellular domains upon binding of neurotrophins (Vilar et al 2009a, b). Activation of p75NTR involves association of a neurotrophin dimer with cystein-rich domains 2-4 of the two extracellular domains of p75NTR dimers (He and Garcia 2004). However, p75NTR can also bind neurotrophins in a complex with the Trk receptors (Chao and Hempstead 1995), and when the two are co-expressed they indeed do form complexes, presumably altering the signaling (Huang and Reichardt 2003).

Although the p75NTR receptor is not the only receptor that can be activated by proforms of neurotrophins: it has been shown that not all p75NTR-expressing cells respond to proNGF. Nykjaer et al. (2004) showed that in terms of proNGF binding, sortilin is involved in influencing cell death at the time of p75NTR binding. Sortilin is a 95kDA receptor of neurotensin that is expressed in areas where NGF and proNGF have an influence. proNGF is able to create a signalling complex by simultaneously binding both p75NTR as well as sortilin. The same effect was shown in other neurotrophins as well, such as proBDNF, where using a competitive antagonist of sortilin blocked sympathetic neuron death. Same effect was seen in neurons deficient in p75NTR (Teng et al. 2005). This means that both p75NTR and its co-receptor sortilin are important in mediating apoptosis by pro-neurotrophin signaling. The proBDNF (and BDNF)-induced apoptosis of sympathetic neurons was accompanied by gamma-secretase-mediated cleavage of p75NTR (Kenchappa et al. 2006). Cleavage of p75NTR was shown to be crucial for this receptor to exert its function, for preventing the action of gamma-secretase inhibited apoptosis. Furthermore, Lee et al. (1994) showed that sympathetic neurons in animals that lacked the p75NTR receptor were more responsive to NT3 than their wild type counterparts. Not only that, but high doses of neurotrophins were also found to

elicit cell death through p75NTR (Lee et al. 2001b). This suggests that the presence of p75NTR decreases the specificity of neurotrophins to their Trk receptors, which can then be increased again upon the removal of p75NTR.

Trk receptors

The structural organization of Trk receptors is conserved amongst all receptors of this class. The extracellular parts of these receptors contain three tandem leucine-rich repeats flanked by two cystine clusters followed by two immunoglobin-like domains type C2 (Ig-C2) (Barbacid 1995, Huang and Reichardt 2003). The second Ig-C2-like domain at which one of its cysteine residues has been replaced by a leucine (Barbacid 1995), is responsible for ligand binding for the large part (Perez et al. 1995) and determines the ligand specificity (Urfer et al. 1995). Although other parts of the receptor influence binding as well as it was demonstrated that the first Ig-C2 domain is required for TrkB binding to its ligands (Urfer et al. 1995, Zaccaro et al. 2001). Trk receptors have also a single transmembrane domain and a single tyrosine kinase domain followed by several tyrosine-containing motifs. Ig-domains are also important in regulating receptor dimerization, a pre-requisite for ligand binding, as it was shown that deletion of one or both of these domains prevents dimerization of the receptors (Arevalo et al. 2000). Receptor specificity, on the other hand, is determined by the N-termini of the receptors (Huang and Reichardt 2003).

There are three main Trk receptors that are responsible for triggering signaling pathways as a response to neurotrophins actions: TrkA, TrkB and TrkC. As mentioned previously, each one of these receptors binds specific neurotrophins.

Differential splicing of the mRNAs encoding each of the Trk receptors has been shown to regulate the specificity of the Trk receptors to some extent, however, such as that of TrkB, for instance, is influenced by presence or absence of a short sequence of amino acids. An isoform lacking this insert is only activated by BDNF, whereas it can respond to NT3 and NT4 as well when the insert is present

(Strohmaier et al. 1996, Boeshore et al. 1999). This is important because of differential expression of these isoforms in different subpopulations of sensory neurons (Huang and Reichardt 2003). This suggests that differential splicing of this receptor plays a crucial role in development.

p75NTR also influences the binding of Trk receptors to their ligands. For instance in its presence TrkB binds NT3 and NT4 with a lower affinity, something that is seen in the case of TrkA as well (in terms of binding NT3) (Bibel et al. 1999, Lee et al. 1994, Huang and Reichardt 2003).

Endocytosis has been shown to play a role in Trk signalling as well. Endocytosis and retrograde transport of vesicles that contain neurotrophins and Trk complexes can be viewed as an important feature by which signalling events at the distant axon terminus control nuclear transcription (Schecterson and Bothwell 2010, Ye et al. 2003). In the case of Trk receptors, internalization is promoted by ubiquitination (Arevalo et al. 2006, Arevalo and Wu 2006).

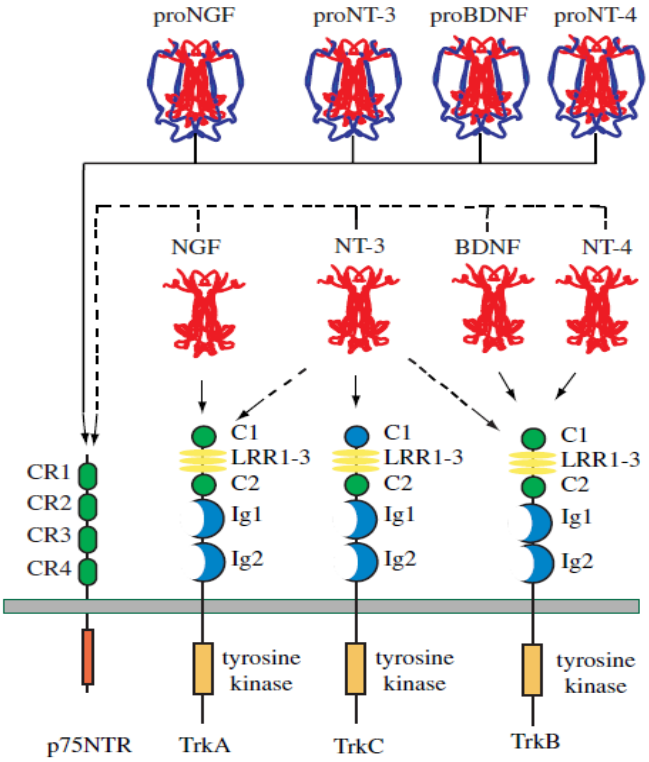

Figure 1: Neurotrophins and their receptors. All pro-forms of neurotrophins are able to activate p75NTR. In their mature form, neurotrophins are more specific: NGF activates TrkA, NT3 preferentially activates TrkC, but is also able to activate TrkA and TrkB; BDNF and NT4 activate TrkB. Adapted from Reichardt 2006.

Trk transactivation

Neurotrophins, however, aren't the only substances that are able to activate Trk receptors. Trk receptors can also be activated by G-protein coupled receptors (GPCRs), adenosine A2A and PAC-1 receptors (Lee et al. 2001, 2002, Huang and Reichardt 2003), although the mechanisms of activation are completely different to those of neurotrophins. The difference is that while neurotrophins activate Trk receptors that are present in the plasma membrane only, these GPCRs can activate, or at least initiate activation, before the Trk proteins even leave the Golgi apparatus (Schecterson and Bothwell 2010), which can then influence the transport of these receptors to the membrane itself (Rajagopal and Chao 2006). There are also other compounds that associate with Trk receptors: ephrin-A5 has been found to associate with TrkB and promote BDNF axon branching (Marler et al. 2008). Furthermore, low-density lipoprotein receptor-related protein 1 (LRP1) also induces transactivation of Trk receptors (Shi et al. 2009), as does zinc ion (Huang et al. 2008c). Both of these factors are dependent on activation of Fyn, which also mediates transactivation of Trk receptors by GPCRs (Huang et al. 2008c, Rajagopal and Chao 2006). This is important from the perspective of time of receptor activation, as it has been shown that the time-dependence of Trk transactivation varies dramatically with co-transactivators (Schecterson and Bothwell 2010), for instance zinc ion activates Fyn and TrkB much faster than PAC-1 (Huang et al. 2008c).

Furthermore, Trk receptors occasionally don't need ligands at all to be activated as it has been shown that high local density of Trk receptors may trigger spontaneous receptor activation even without the presence of any ligands (Schecterson and Bothwell 2010).

1.1.3 The role of neurotrophins and their receptors in development: neuronal survival

The activation of neurotrophin receptors by their ligands plays a crucial role in the survival of neurons in most ganglia throughout the peripheral nervous system, and, to a lesser extent in the central nervous system as well. Interestingly, neurons in the PNS show high dependency on specific combination of one or more neurotrophins, while in the CNS the neurons are less susceptible to a loss of single neurotrophin, suggesting dependency on more than one, or a combination, of different neurotrophins (Bibel and Barde, 2000).

In the PNS, activating the Trk receptors plays an important role in supporting the survival of the sensory neurons primarily in the dorsal root, vestibular and cochlear, trigeminal, geniculate, and the nodose-petrosal ganglia.

The DRG contains several subpopulations of neurons that are dependent on single neurotrophins. Farinas et al. (1994) showed that null mutation of *Nt3* and *TrkC* caused a loss of proprioceptive neurons in this ganglion. Null mutation of *Ngf* and *TrkA*, on the other hand, caused an extensive loss of the nociceptive DRG neurons, located in the fourth and fifth lumbar region of this ganglion (Crowley et al. 1994, Smeyne et al. 1994).

The survival of neurons in the vestibular ganglion is mostly dependent on BDNF-TrkB signaling (Ernfors et al. 1994, Jones et al. 1994).

Similarly to the DRG, the trigeminal ganglion contains subpopulations of neurons that are dependent on different single neurotrophin. Neurons in this ganglion are partially lost in absence of either BDNF-TrkB signalling, NT3-TrkC signalling and NGF-TrkA signalling (Ernfors et al. 1994, Farinas et al. 1994, Smeyne et al. 1994).

Analogous neuronal dependency on neurotrophins was shown in the nodose-petrosal and the geniculate ganglion: neurons in these ganglia were shown to be dependent on either BDNF or NT4, as single mutations caused a loss of about half of the neuronal population, while the double *Bdnf/Nt4* mutation and the *TrkB* null mutation caused a loss of nearly all (90-95%) neurons (the geniculate ganglion

will be discussed in more detail in Chapter 2) (Conover et al. 1995, Liu et al. 1995, Minichiello et al. 1998). A study using a triple *Nt3/Nt4/Bdnf* mutant showed that nearly all of the neurons in the geniculate ganglion were lost, as were all neurons in the vestibular ganglion and a large majority of neurons in the trigeminal and nodose-petrosal ganglia, further supporting the notion of subpopulations of neurons in these ganglia being dependent on separate single neurotrophins in the peripheral nervous system (Liu and Jaenisch 2000).

In the central nervous system, however, the situation is quite different. Studies using single neurotrophin mutations found the cranial ganglia largely unaffected: *Bdnf* null mutation did not cause any loss in facial motor neurons, dopaminergic neurons of the substantia nigra or neurons in the basal forebrain (Jones et al. 1994). *Ngf*, *Nt3* and *Nt4* mutations were not found to have any marked decrease of neuronal populations in the central nervous system, either (Conover et al. 1994, Farinas et al. 1994, Smeyne et al. 1994,). It has been therefore hypothesized that neurons in the central nervous system are capable of surviving on a combination of specific neurotrophins as they appear to survive in absence of a single neurotrophins or their respective receptors. There are, however, populations that do not appear to be responsive to even to a combination of neurotrophins, as for instance a triple mutant of *Nt3/Nt4/Bdnf* caused only a small decrease of spinal and facial motor neurons (Liu and Jaenisch 2000).

Most CNS neurons express TrkB and TrkC, while TrkA expression is very restricted (Holtzman 1992). The important roles of these receptors were supported by studies showing the dentate gyrus and cerebellar granule neurons lost in *Trkb* mutant mice and, to a larger extent, *Trkb/Trkc* double mutant mice (Alcantra et al. 1997, Minichiello and Klein, 1996), suggesting combinatorial or cooperative support of neurotrophins for the neurons in the central nervous system.

Neurotrophins are also crucial in influencing the innervation patterns of the fibers from the ganglia, for example the pathways used by axons from the geniculate ganglion into the tongue; however, this will be discussed in detail in Chapter 2.

Because my project was focused on TrkB, the rest of the thesis will be concerned about TrkB only, and will thus be discussing the activation and signalling pathways associated primarily with this receptor.

1.1.4 TrkB signalling pathways

TrkB activation occurs via a two-step process, where at first there is ligand-mediated oligomerization of receptor molecules at the cell surface, which is followed by autophosphorylation of the tyrosine residues (Barbacid 1995).

These intracellular tyrosine residues are present in the intracellular kinase domain of the receptor and they act as anchors for binding two classes of downstream signalling molecules; adaptors and enzymes. Phosphorylation of these domains leads to an open conformation of the receptor which results in trans-phosphorylation and allowing the substrates to access the kinase. Thus, phosphorylation regulates the tyrosine kinase activity and so provides phosphorylation-dependent trigger for initiation of intracellular signalling pathways (Huang and Reichardt 2003, Bibel and Barde 2000).

There are several tyrosine residues in the juxtamembrane domain of the carboxyl terminus of this receptor that can act as docking sites for proteins that contain phosphotyrosine binding domains (PTB) or Src-homology 2 domains (SH2) (Huang and Reichardt 2003). In the case of TrkB, tyrosine at position 515 is able to recruit SHC adaptor molecules as well as fibroblast growth factor receptor substrate 2 (FRS2) via PTB domain (Kavanaugh and Williams 1994), which activate Ras-mitogen-activated protein kinase (MAPK) signalling pathway as well as the phosphatidylinositol 3-kinase (PI3K) cascade.

The second important adaptor site is at the tyrosine position 816 in the C terminus. Phosphorylation of this site causes recruitment of phospholipase Cγ (PLCγ), which binds via a Src-homology 2 (SH2) domain, followed by phosphorylation of PLCγ. This results in generation of inositol-1,4,5-trisphosphate (Ins(1,4,5)P3) and diaglycerol (DAG) (Huang and Reichardt 2003, Minichiello 2009).

There are additional adaptor proteins that are able to bind the TrkB receptor, containing pleckstrin homology (PH) and SH2 domains (such as SH2B2), and cause its activation (Bibel and Barde 2000). A simplified version of the TrkB signalling pathways is depicted in Figure 2.

RAS-MAPK pathway

Activation of this pathway downstream of the TrkB receptor is important for neuronal differentiation as well as survival for many neuronal subpopulations (Reichardt 2006).

As previously mentioned, recruitment of adaptor proteins SHC or FRS2 causes transient activation of Ras (a GTPase). Phosphorylation of SHC of FRS2 creates a phosphotyrosine site on the adaptor protein (SH3 domain) that recruits GRB2 (growth factor receptor-bound protein 2) bound to the Ras exchange factor Son of Sevenless (SOS, a guanine nucleotide exchange factor (GEF)), which then activates Ras. Activated Ras stimulates signalling through several factors such as PI3K (discussed below) and c-Raf-Erk (Huang and Reichardt 2003, Reichardt 2006) and p38MAP (Xing et al.1998).

Phosphorylation of Raf (serine/threonine kinase) activates ERK1, ERK2 (extracellular signal-regulated kinase) by phosphorylation of MEK1 and/or MEK2 (MAP kinase kinase). ERK5 is also phosphorylated by Raf through sequential activation of MEKK3 (MAP kinase kinase kinase 3) and MEK5 (Esparis-Ogando et al. 2002, Sun et al. 2001). This then in turn activates RSK (ribosomal protein S6 kinase). RSK and MAP kinase-activated protein kinase-2 can phosphorylate CREB (cAMP responsive element binding protein), a protein that has been shown to regulate transcription of genes whose products influence differentiation and survival of neurons (Lonze et al. 2002, Riccio et al. 1999).

Ras also activates p38MAP kinase which involves sequential activation of RalGDS (Ral guanine nucleotide dissociation stimulator), Ral and Src (Huang and Reichardt 2003). p38MAP then activates MAP kinase-activated protein kinase-2,

which activates CREB as well. Termination of signalling of this pathway is caused by ERK- and RSK-mediated phosphorylation of SOS, which dissociates the SOS-GRB2 complex (Kao et al. 2001).

All of the signalling described above leads to transient activation of these signalling pathways, namely the MAPK signalling. There are, however, other factors that may lead to prolonged signalling. This function appears to require recruitment of and phosphorylation of FRS2, which in turn provides recruitment sites for adaptor proteins such as GRB2, CRK (CT-19 related kinase), Src and SH-PTP2 (protein-tyrosine phosphatase C2) (Meakin et al. 1999, Kao et al. 2001). In this case the Rap1 exchange factor C3G (a GEF) is activated, phosphorylating Rap1 (Ras-related protein). This triggers the Erk kinase cascade which causes sustained MAP kinase activation by (likely) removal of an inhibitory factor (Reichardt 2006). There are also additional adaptors that are able to influence the above-described signaling. Adaptors like rAPS and SH2-B are phosphorylated following Trk activation and have been found to form complexes with GRB2 and SOS (Qian and Ginty 2001, Rui et al. 1999) and can also lead to sustained activation of the MAPK signalling pathway.

Furthermore it has been shown as well that not only the duration but also the strength of this signalling can be modulated. Patterson et al. (2001) showed that by altering the subcellular distribution and nuclear translocation of MAP kinases, the signal strength can be amended.

PI3K pathway

Binding of the SHC adaptor protein at position Y515 can also activate PI3K signalling pathway, which is important for regulation of neuronal survival of several different population of neurons (Bibel and Barde 2000, Huang and Reichardt 2003). PI3K can be recruited via Ras-dependent or Ras-independent pathways. First, phosphorylation at the SHC adaptor site can phosphorylate GRB2 bound to SOS, which then activates Ras, subsequently activating PI3K. Second, GRB2 can also

activate adaptor proteins GAB1 and GAB2 which then leads to phosphorylation of PI3K as well (Holgado-Madruga et al. 1997, Reichardt 2006). Third, PI3K can also be activated by Trk-dependent phosphorylation of IRS1/2 (insulin receptor substrate 1/2) (Yamada et al. 1997).

Upon activation, PI3K generates phosphoinositides such as PIP (phosphatidylinositol phosphate), PIP2 (phosphatidylinositol bisphosphate) and PIP3 (phosphatidylinositol trisphosphate), which activate PDPK1 (3-phosphoinositide-dependent protein kinase 1). PDPK1 together with 3-phosphoinositides then activate protein kinase Akt (protein kinase B). Akt is able to control activities of several proteins that are crucial for cell survival (Yuan et al. 2003, Reichardt 2006, Minichiello 2009). One of such proteins is BAD, a Bcl2-family member protein that regulates apoptosis through sequestration of Bcl-XL, a protein involved in inhibition of Bax (a pro-apoptotic protein) (Datta et al. 1997, Huang and Reichardt 2003). Akt also phosphorylates FKHRL1, a transcription factor that is involved in regulation of proapoptotic genes (Brunet et al. 1999). Both FKHRL1 and BAD are sequestered by 14-3-3 proteins upon phosphorylation by Akt, which prevents them from promoting apoptosis (Datta et al. 2000, Brunet et al. 2001).

Furthermore, Akt phosphorylates IκB, which is subsequently degraded and thus promotes release of NF-κB. NF-κB has been shown to promote sensory neuron survival (Maggirwar et al. 1998, Hamanoue et al. 1999). Lastly, Akt phorphorylates the S6 kinase important for promoting translation of mRNAs (Kimball et al. 2002).

PI3K-generated phosphoinositides can also recruit signalling molecules such as GEFs for Rho proteins, Cdc42 and Rac, who then control organization of the F-actin cytoskeleton, involved in growth cone steering (Yuan et al. 2003).

PLCγ signalling

As mentioned previously, PLCγ1 is recruited to the tyrosine position at 816 on the C-terminus domain of the TrkB receptor. Once docked, PLCγ1 is phosphorylated and then hydrolyzes phosphatidylionsitol 4,5-bisphosphate (PtdIns (4,5)P2) to create inositol 1,4,5-trisphosphate (IP3) and diaglycerol (DAG) (Reichardt 2006, Minichiello 2009).

IP3 causes Ca^{2+} release from cytoplasmic stores into the cytoplasm by docking at the IP3-gated Ca^{2+}-release channels. This calcium then activates Ca^{2+}/calmodulin-dependent protein kinase II and IV (CaMKII and CaMKIV). CaMKs have been implicated in influencing synaptic plasticity long term potentiation (LTP) and learning. CaMKII has been shown to be important for early long-term potentiation (E-LTP) as the induction of E-LTP requires short-term activation of CaMKII (and PKC), although maintenance is defined by the continual activation of these enzymes (by autophosphorylation) (Soderling 2000, Lisman et al. 2002, Minichiello 2009). Its actions are exerted via phosphorylating PSD proteins like GluR1 subunit of the AMPA receptor and the NMDA receptor. CaMKIV, on the other hand, is able to phosphorylate CREB, and thus inducing transcription of genes involved in survival, neural differentiation and synaptic plasticity (Finkbeiner et al. 1997). Minichiello et al. 2002 showed that regulation of synaptic plasticity and LTP via CREB-induced transcription was dependent on activation of the PLCγ1 signalling pathways, but not the MAPK-signalling pathways.

The production of DAG stimulates DAG-regulated isoforms of protein kinase C. This pathway has been implicated in influencing synaptic plasticity and learning (Saito and Shirai 2002), however, it is yet not clear whether this pathway is specifically activated by TrkB signalling.

There are also other proteins activated by PLCγ1, such as PKCδ (protein kinase C-δ), which is involved in activation of ERK1/2 and MEK1 in Ras-independent way (Corbit et al. 1999).

Short term receptor activation

The duration of TrkB receptor activation has been shown to elicit different cellular responses. While constitutive release of BDNF and NT4 leads to prolonged activation of the downstream TrKB receptor signalling pathways, acute release of these ligands can trigger short-term upregulation of proteins associated with these signalling pathways (Balkowiec et al. 2002, Goodman et al. 1996, Griesbeck et al. 1999, Lessmann et al. 2003). The different effects of long- and short-term TrkB activation has been shown by Ji et al. (2009), who demonstrated that responses elicited by both gradual and acute application of BDNF to hippocampal slices elicited prolonged and transient activation of TrkB, respectively. In turn, this led to enhancement of basal synaptic transmission when the TrkB receptor was activated transiently, while long-term potentiation (LTP) was stimulated only when TrkB was stimulated gradually.

LTP can be divided into three sequential events: short-term potentiation (STP), early long-term potentiation (E-LTP) and late long-term potentiation (L-LTP). Together with the early phases of long term potentiation, STP is independent of gene transcription, and, by definition, transient (Sweatt 1999, Kandel 2001). Transient activation of TrkB is required for both STP and E-LTP, where enzymes downstream of TrkB signalling, particularly Ca^{2+}/calmodulin-dependent protein kinase II (CamKII) and potein kinase C (PKC), are triggered by a rapid rise in intracellular Ca^{2+} concentration that is associated with mediating of the induction of E-LTP (Minichiello 2009). BDNF is crucially important for this process as it has been shown that deleting *Bdnf* disrupted normal induction of E-LTP in hippocampal slices of young mice (Korte et al. 1995), and inhibition of BDNF binding to TrkB reduced synaptic responses to high frequency stimulation and magnitude of E-LTP (Figurov et al. 1996). The transient activation here is distinguished from maintenance of LTP, where the Ca^{2+} influx is not longer required and enzymes such as CamKII and PKC become autonomously active. These processes, together with resulting synaptic consolidation, are involved in memory formation (Minichiello 2009).

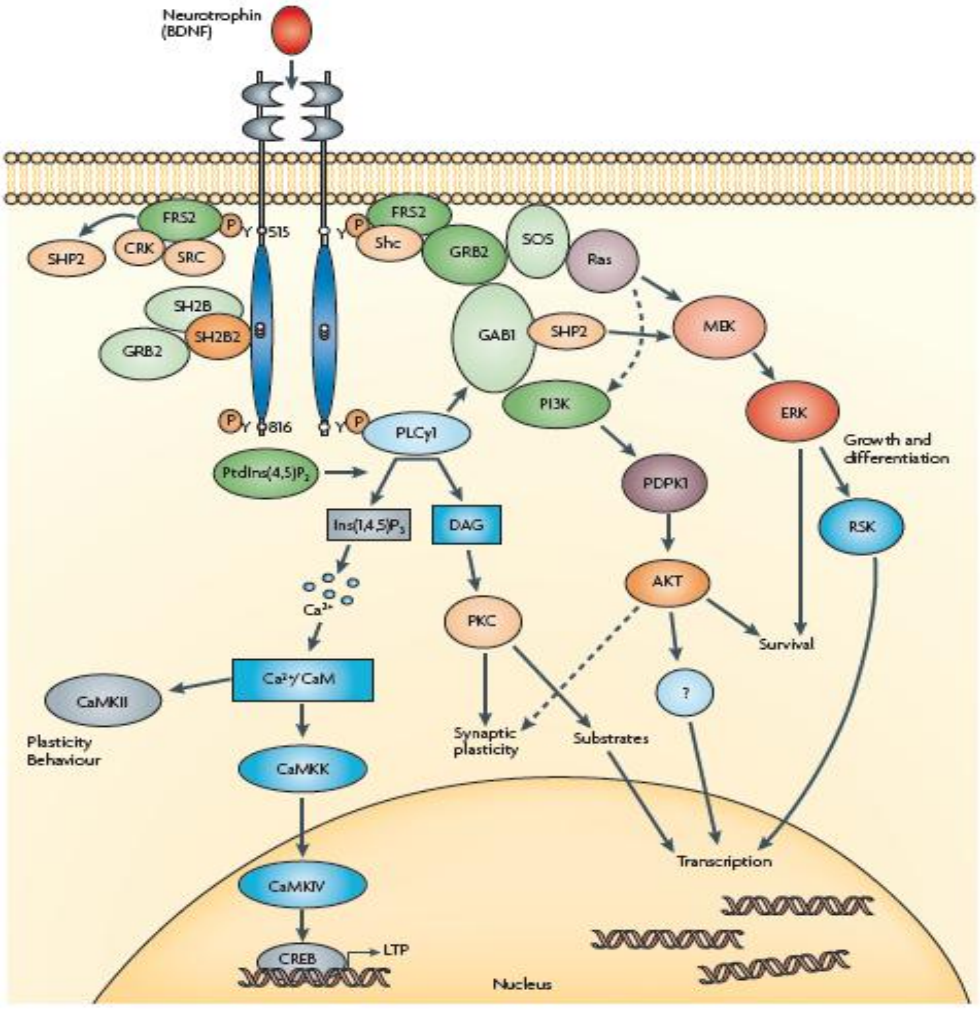

Figure 2: Signalling pathways downstream of TrkB. Binding of either BDNF or NT4 leads to receptor dimerization and subsequent phosphorylation of its adaptor site. Activation of Shc adaptor protein at Y515 activates MAPK cascade leading to differentiation and growth and recruitment of ERK and MEK factors. PLCγ1 at Y816, when activated, generates (Ins(1,4,5)P_3), leading to intercellular Ca^{2+} release and stimulation of CaM/Ca^{2+}-dependent kinases. Activation of PLCγ1 can also lead to activation of DAG and PKC. Image taken from Minichiello (2009).

1.2 Rodent gustatory system

1.2.1 Structure of the taste system

Rodent gustatory system is a specialized chemosensory system that is used by animals for evaluating nutrition. Using this system, incoming compounds can be evaluated in terms of their value as nutritional or harmful and based on this they can then be either accepted or rejected as a response of innate behaviour. Even though it has been shown that vertebrates and insects have evolved this system independently, there are many similarities between them in terms of organization and the way the signals are transferred between the receptors and processing areas in the brain, also referred to as the coding logic (Yarmolinsky et al. 2009).

There are five different primary taste qualities (also called modalities): sweet, bitter, sour, salty and umami (Chandrashekar et al. 2006). These five can be further divided based upon their hedonic value into “good” such as sweet and umami, which signal nutritional value and trigger ingestion; and “bad” such as bitter and sour, which signal presence of potentially harmful substances and thus trigger rejection. Salt is quite unique in a sense that it can be viewed as both “good” and “bad” according to the concentration and physical needs of the particular animal (Lindenmann 2001, Bachmanov et al. 2002). The precise organization of these primary tastes according to their hedonic qualities is beyond the scope of this thesis.

In rodents, taste qualities are detected by taste receptor cells (TRCs) present on the tongue and the palate. TRCs are organized into taste buds, onion-shaped aggregates of about 50-100 cells (Kinnamon et al. 1993, Lindemann 2001). Taste buds are then organized into structures called papillae, which are comprised of an epithelial layer over a centre of connective tissue (Jung et al. 2004). These are located on the tongue, in the nasoincisive papilla and in the eminences on the soft palate (Krimm 2007). There are three different types of gustatory papillae: fungiform, foliate and circumvallate papillae; all of these contain taste buds. The

fungiform papillae are located on the oral part of the tongue and are organised in a very specific patterned array, with the large majority of the papillae located on the anterior-most part of the tongue. There is only one circumvallate papilla in rodents, and that is found in the middle part of the terminal sulcus (Jung et al. 2004), while the foliate papillae are located in ridges located in the caudolateral part of the tongue. There is also another type of papillae, the filiform papillae, cone-shaped structures which cover most of the tongue, but do not contain TRCs. Instead, they possess a core of connective tissue covered by an epithelium that expresses hair-related keratins (Dhouailly et al. 1989, Mbiene and Mistretta 1997, Jung et al. 2004) and contain receptors for somatosensory signalling (Fan et al. 2004, Oakley et al. 2004).

Taste buds are innervated by sensory neurons (cranial nerves) of several ganglia: lingual afferents from the geniculate ganglion innervate taste buds that are present on the anterior two thirds of the tongue via the chorda tympani nerve, and on the palate via the greater superficial petrosal nerve. The geniculate ganglion has also been found to facilitate innervation into the outer ear; however, this will not be discussed in this thesis (Krimm 2007, Yarmolinsky et al. 2009). Neurons from the petrosal ganglion innervate the circumvallate papilla and the taste buds in the foliate papillae, via the glossopharyngeal nerve (Figure 3). The epithelium around the taste papillae is also innervated by a trigeminal ganglion, which, however does not contribute to the innervation of the taste buds themselves (Mbiene 2004).

The cranial nerves therefore connect the taste buds located on the tongue and the palate with the geniculate and petrosal ganglia. From these ganglia, the three nerves continue into the rostral nucleus of the solitary tract (NST) in the medulla where they form distinct, but overlapping terminal fields (May and Hill 2006). From here the taste responses are transmitted into the parabrachial nucleus, into the thalamus until they reach the primary gustatory cortex in the insula (Yarmolinsky et al. 2009) (Figure 3). During development the fields undergo morphological development, with the chorda tympani undergoing the greatest change (Sollars 2005, Sollars et al. 2006). In rat, for instance, the terminal field of

this nerve has been shown to first increase during development, only to be followed by a decrease of around 50% between postnatal days P15 and P25, and then decrease again by further 40% by P35, after which it remains constant (Mangold and Hill 2008). Even though the size of the terminal fields varies with age, the amount of overlap between the nerves does not change (Corson and Hill 2011).

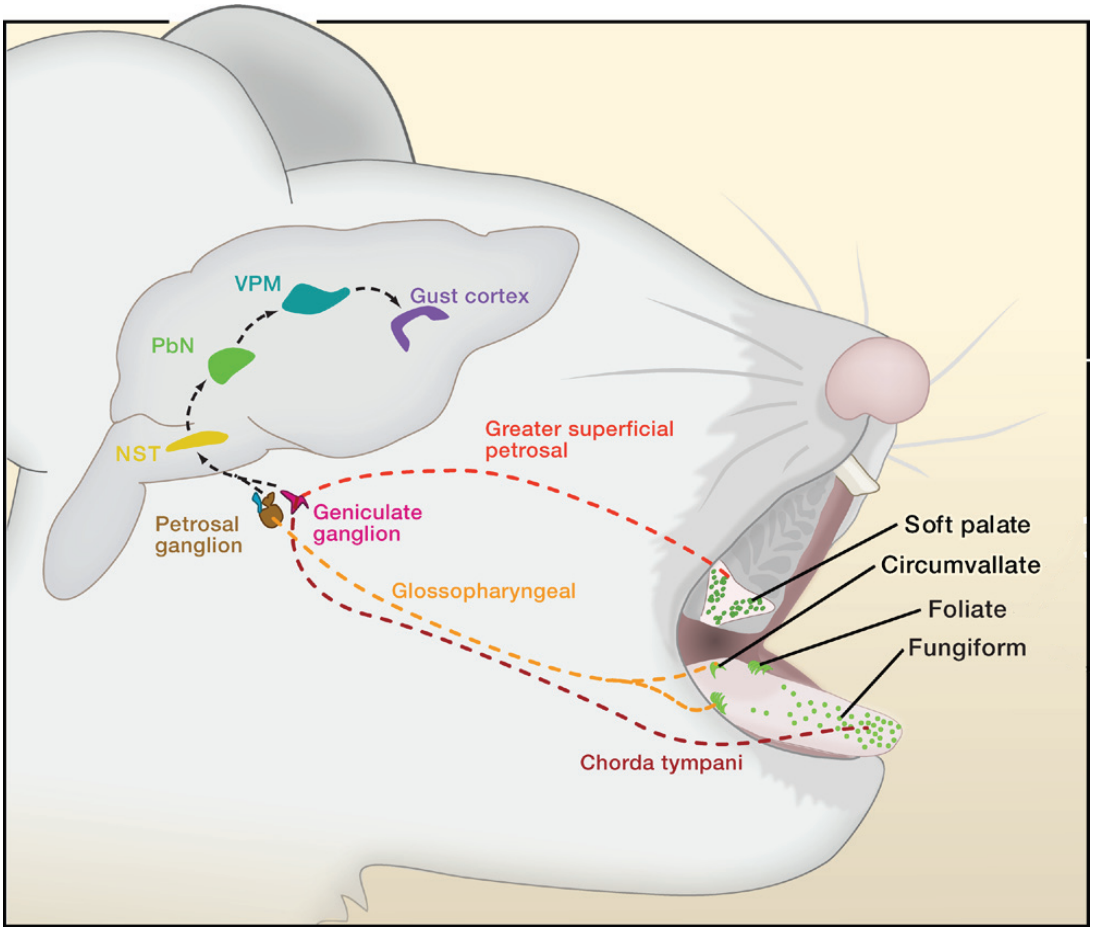

Figure 3: Rodent gustatory system overview. Taste buds present on the anterior tongue and the soft palate are innervated by chorda tympani and greater superficial petrosal nerves connecting to the geniculate ganglion. Taste buds in the circumvallate and foliate papillae are innervated by the glossopharyngeal nerve connecting to the petrosal ganglion. From there the taste signals are transmitted into the nucleus of the solitary tract (NST), parabrachial nucleus (PbN), ventral postero-medial nucleus of the thalamus (VPM) until they reach the gustatory cortex in the inula. Adapted from Yarmolinsky et al. (2009).

1.2.2 Facilitating taste

Taste receptor cells

The apical ends of the taste receptor cells on the tongue extend microvilli into the taste pore where the cells get exposed to the particular tastants. Once the taste receptor cells are exposed, only receptors specifically adapted to detect these tastants are triggered (Bear et al. 2007). Each taste receptor cell has a lifespan of about two weeks, creating a constant cycle of renewal and growth of these cells. There are three main types of TRCs: Type I, Type II and Type III (Figure 4).

Type I cells are the most common cells in taste buds. They express GLAST, a glutamate transporter, as well as NTPDase 2, a plasma membrane-bound nucleotidase hydrolyzing extracellular ATP (Bartel 2006). Type I cells are therefore deemed to perform a role similar to those of glial cells in the central nervous system in a way that they restrict the spread of transmitter. Interestingly, Type I cells have shown ionic currents that have previously been implicated in salt taste transduction (Vandenbeuch et al. 2008), thus adding to the salt taste controversy, which will be discussed later.

Type II (receptor) cells have been shown to contain receptors that are involved in detecting sweet, bitter and umami tastants. These cells express voltage-gated Na^+ and K^+ channels together with hemichannel subunits so that they can be key players in taste-evoked secretion of ATP (Chaudhari and Roper 2010). Importantly, Type II cells have been shown to be specialized, or "tuned" to a specific taste, so that any given Type II TRC will only respond to either sweet, bitter, or umami tastants.

Type III (presynaptic) cells form synaptic junctions with nerve terminals. They express NCAM and voltage-gated Ca^{2+} channels typically associated with neurotransmitter release (Dvoryanchikov et al. 2007). Like Type II cells, Type III cells also express a set of voltage-gated Na^+ and K^+ channels (Vandenbeuch and Kinnamon 2009). Importantly, Type III cells respond directly to sour tastants as well as to carbonated solutions, however, are not limited to these (Huang et al. 2008b,

Tomchik et al. 2007). Differently to Type II cells, Type III cells are not specialized to single specific tastants, but respond to a broad range of compounds (Tomchik et al. 2007).

There is also a fourth type of cell that is generally found in taste buds. These are basal cells that do not extend processes into the taste pores. It is not yet clear whether the basal cells represent undifferentiated cells, or what the actual importance of these cells is, however, they appear to be an ever-present part of the taste bud (Chaudhari and Roper 2010).

Finally, taste buds also contain marginal cells (Type V in Figure 4), that are thought to play a role in cell migration and taste cell differentiation (Witt and Reutter 1998, Doty 2012).

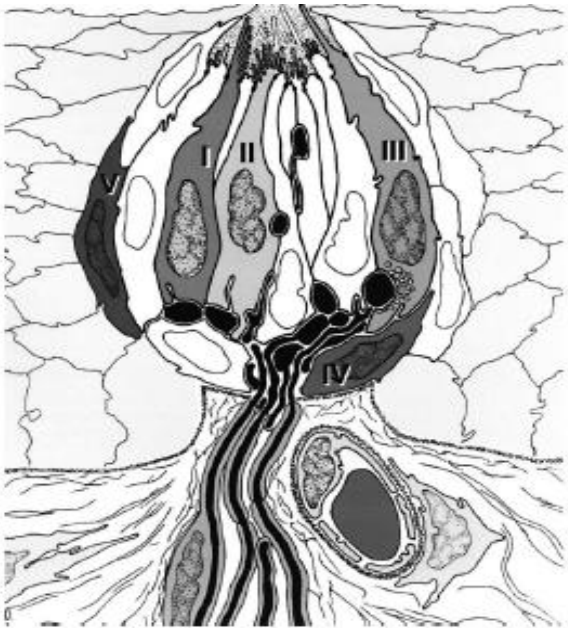

Figure 4: Idealized diagram of a taste bud. Each taste bud contains several types of cells with different properties. Types I, II and III elongated taste cells that are used for detecting tastants: Type I is glial-like cell, Type II receptor cell and Type III presynaptic cell. In the diagram cells labelled IV are basal cells and V marginal cells. Adapted from Doty (2012).

Receptors for tasting

Alongside the differences in processing of tastants according to their hedonic value, there are also differences in processing on a more fundamental level than the taste receptor cells; it is the very receptors present on these cells that are involved in detecting these tastants that are different.

The pleasant, attractive tastes are sensed by heterodimeric G protein-coupled receptors (GPCRs) (Yarmolinsky et al. 2009), that are assembled in several different combinational arrangements of T1R1, T1R2 and T1R3 subunits (Nelson et al. 2001, Li et al. 2002, Zhao et al. 2003). More specifically, umami is detected by a heteromeric receptor composed of the T1R1 and T1R3 subunits (Nelson et al. 2002, Zhao et al. 2003), whereas sweet taste is detected by a combination of T1R2 and T1R3 subunits. The sweet receptor is interesting for its ability to recognize a wide combination of sweet tastes including sugars, artificial sweeteners as well as D-amino acids (Yarmolinsky et al. 2009). The receptors that are involved in tasting pleasant and nutritious food have been shown to be low-affinity receptors (Damak et al. 2003), presumably due to the need of the animal to recognize sugar levels above nutritionally-relevant levels. This, however, is not the case with bitter substances. Because they are potentially toxic, animals need a system that is able to identify even a presence of miniscule amounts of these substances. For this reason, bitter substances are detected by T2R subunits of the GPCRs (Chandrashekar et al. 2000). About 40 different types of T2R subunits have been found previously (depending on the species), and each one of the subunits is able to detect vast variety of bitter substances (Meyerhof et al. 2010). Sour taste is detected by PKD2L1, a TRP ion channel (Huang et al. 2008b). Although there have been other possibilities for the acid sensors, such as PKD1L3, HCN1 and HCN 4, as well (Ishimaru et al. 2006, Huang et al. 2008b). Carbonation, which can be defined as the presence of CO_2, has also been found to be detected by mammalian taste receptors; this has been found to be facilitated by membrane-anchored carbonic anhydrase IV (Chandrashekar et al. 2009).

The identity and functionality of the salty taste receptor has not been completely understood yet. Because behavioural experiments have shown that the response of mice varies depending on the concentration of NaCl in solution (mice would consume low-salt solutions but avoid high-salt containing solutions) (Bachmanov et al. 2002), and this behaviour was shown to be blocked by ENaC (epithelial sodium channel) channel blocker amiloride, it was postulated that it is the ENaC that is involved in salt detection. ENaC is quite different to the voltage gated Na^+ channel in that it does not respond to changes of voltage; instead, it stays open at all times (Bear et al. 2007). Recently, Oka et al. (2013) showed that the aversive behaviour associated with high quantities of salt may be caused by a recruitment of two primary aversive taste pathways that are usually activated as a response to sour and bitter tastants. However, whether this is the only receptor dedicated for salt detection remains to be seen.

Taste signalling

Receptor (Type II) cells and presynaptic (Type III) cells release different neurotransmitters (Huang et al. 2007). Receptor cells release ATP via pannexin channels upon stimulation, which is thought to be an important excitatory transmitter between the TRCs and the gustatory fibers leading to the geniculate. Presynaptic cells, on the other hand release serotonin, noradrenaline and GABA (Dvoryanchikov et al. 2007, Chaudhari and Roper 2010).

Upon entering the tongue, different tastants are able to excite the taste buds present on either the tongue or the soft palate. When this happens, ATP secreted from the receptor cells stimulates the lingual afferents, and at the same time excites the adjacent presynaptic cells which then release serotonin and/or noradrenaline. Furthermore, ATP can also stimulate the receptor cells themselves, thus increasing its own secretion (Huang et al. 2008a, 2009).

The release of serotonin, however, appears to have the opposite effect to that of ATP in a way that the release of serotonin inhibits the receptor cells rather

than exciting them. This, together with the excitatory stimulation by ATP seems to shape the final signal from the tastants that is sent by the lingual afferents further along the gustatory pathways into the gustatory ganglia and the hindbrain (Chaudhari and Roper 2010).

Recent experiments showed involvement of glutamatergic signalling in taste buds as well. Huang et al. (2012) showed that many of the presynaptic cells were able to respond to NMDA as well as kainic acid (AMPA receptor agonist), suggesting the possibility of synaptic glutamate modification of signal output from taste buds.

Coding of taste

As mentioned previously, each one of the tastes is detected by specialized receptors on taste receptor cells. There has, however, been some ambiguity in the past about how each of the tastes is coded for. The most prevailing theories suggested two possibilities; one where the taste receptor cells could detect multiple taste qualities, and a second one where the taste buds were able to detect only one taste quality, but the afferents would carry information for multiple taste qualities (Smith and St. John 1999, Erickson 2000). Both of these models became known as "across-fibre pattern of coding", implicating that the tastant recognition stems from the combined activity of different classes of these taste receptor cells (Chandrashekar et al. 2006).

Over the past few years it has become more apparent, that each receptor class is expressed in its own distinct taste cell type, thus defining a "one cell, one taste" coding scheme. This system has been subsequently found to be conserved among mammals and it represents the mechanism through which individual taste qualities can be coded on the tongue (Chen et al. 2011, Yarmolinsky et al. 2009, Chandrashekar et al. 2006). It is therefore the "labelled lines" model that at the moment represents the way taste is encoded in the tongue and sent to higher brain areas for interpretation.

Taste representation in the brain

How is taste actually represented in the brain? For a long time the organization of the taste cortex has eluded explanation. Recently, however, studies done by Chen et al. (2011), using two-photon calcium imaging attempted to determine the organization of the gustatory cortex in vivo. Using this method, Chen et al. were able to simultaneously monitor large groups of cells while at the same time have the ability of a single cell resolution. These experiments led to a conclusion that each of the five tastes, which are coded by individual taste receptor cells that are specialized towards a single tastant (although the possibility of existence of a small number of broadly-tuned taste receptor cells was not excluded), are represented in the insula in a precise and spatially ordered gustotopic map. In this case, each taste is represented in its own stereotypical and segregated cortical field. In each of the fields there is a population of neurons that respond to a single specific taste that are not found in other fields. This organization appears to be somewhat different from other sensory systems such as the olfactory, auditory and visual systems where there is an area containing neurons from several populations, thus creating an area of smooth transition between each specialized field (Chen et al. 2011, Tusa et al. 1978). This is, however, not the case in the gustatory system.

These experiments were further supported by multi-photon micro-endoscopy of the geniculate ganglion. By using genetically encoded calcium sensors, Robert Barretto (2012) showed a spatially ordered gustotopic maps present in the geniculate ganglion (where each taste quality was encoded in its own stereotypical cortical field), that included cells of seven categories: five tuned specifically to each of the five basic tastes and two categories of cells that responded to either sweet/umami or bitter/sour (Barretto 2012). Although preliminary, this model provides a very neat explanation of the rodent gustatory system. However, as most models, it also possesses some shortfalls. For instance, it has not been clearly explained what happens in the areas that are not included in any of the taste-specific neuron populations. It has been postulated that perhaps these areas serve

for other aspects of taste coding, such as responding to taste mixes such as the ones shown to be specific to sweet/umami and bitter/sour in the geniculate ganglion, or even integrating other senses with the taste system, however, the definite answers are yet to be obtained.

1.2.3 Development of the taste system

Genesis of the gustatory ganglia: epibranchial placodes

Both the geniculate and petrosal ganglia arise from the epibranchial placodes (which are temporary thickenings of the ectodermal layer in early-stage embryonic mouse), including cells from the neural crest (Krimm 2007). The first placode goes on to develop into the geniculate ganglion, while the second placode develops into the petrosal ganglion.

The development of these placodes is initially dependent on the *Eya* family of transcription factors that are expressed in the pre-placodal region. Together with these, *Six 1/2* and *Six 4/5* have been found to be crucial for the development of both the geniculate and the petrosal ganglia (Zou et al. 2004) at the very first stages of the rising of the placodes in developing ectoderm.

After the expression of *Eya* and *Six* families, influence of *Pax* genes sets off, most importantly *Pax 2* and *Pax 8* that are expressed in the posterior placodal region (Schlosser 2006). Other factors that are crucial for the development of the placodes are signals that arise from the pharyngeal pouch, such as the Bone morphogenic protein family (BMP) (Begbie et al. 1999). The specific members of the BMP that are involved in these processes have not been determined yet.

By embryonic day 9 (E9) in mice, the epibranchial placode cells differentiate into neuroblasts and delaminate. This is followed by a migration and amalgamation into formations of geniculate and petrosal ganglia (Baker and Bronner-Fraser 2001). This process is controlled by basic-helix-loop-helix (bHLH) transcription factors,

most importantly neurogenin 2 (*Ngn2*) in mice. The expression of this gene is dependent on the expression on *Eya1* (Zou et al. 2004). *Ngn1* has not been found to have any major influence on the formation of either the petrosal of geniculate ganglia, but it is involved in influencing the formation of the trigeminal ganglion (as mentioned previously) (Ma et al. 1998). Furthermore, there are also two other transcription factors that influence the formation of these two ganglia at embryonic day E9, the *Phox2a* and *Phox2b* (Zou et al. 2004, Fode et al. 1998). Neurotrophins can also influence the gustatory ganglion neuron phenotypes in terms of neuronal differentiation (Huang and Reichardt 2001).

Once the neuronal precursors have migrated into the target ganglia, they continue to proliferate, causing ganglion expansion (Altman and Bayer 1982). The peak of this expansion happens at roughly E10 in mice, although it continues after this period as well. During the expansion it is typical for neurons in these ganglia to be overproduced, resulting in cell death, which reaches its peak at around E14.5 in mice (Carr et al. 2005). It is at this point that the gustatory afferents reach their fungiform papillae in the tongue and start forming neural buds (Lopez and Krimm 2006, Mbiene 2004, Farbman and Mbiene 1991).

Development of the fungiform papillae

As described previously, fungiform papillae are composed of taste epithelium, surrounding non-taste epithelium and a core of connective tissue that contains sensory fibers that innervate the papillae and the taste bud cells. They are present on the anterior two-thirds of a mouse tongue and are organized in rows that are parallel to the (papilla-free) median furrow (Mbiene and Mistretta 1997, Mistretta et al. 2003, Liu et al. 2012).

Initially, the fungiform papillae arise from epithelium on the tongue at about E11.5-E12 in mouse, and can be then morphologically identified as clusters of cells in focal regions (which are the papillae placodes) about a day later at E13.5 (Mbiene and Roberts 2003). Epithelial cells in the papilla placodes and the early papillae are

mitotically dormant, however, the surrounding areas are not: the lingual epithelium proliferates rapidly (Farbman and Mbiene 1991, Zhou et al. 2006). This causes a series of invaginations and evaginations of the epithelial thickenings that define the placodes, which then form the papillae, including the epithelium encasing the connective tissue core (Mistretta and Liu 2006).

There are many factors that influence the development of fungiform papillae, many of which have been found (in culture experiments) to be retained independently of sensory innervation (Mbiene and Mistretta 1997, Nosrat et al. 2001). The few most important factors discussed here will include Shh (Sonic hedgehog), BMP (bone morphogenic protein) and Noggin (an antagonist to BMP), Wnt, Sox (SRY-related HMG-box) and EGF (epidermal growth factor).

Shh is present throughout the lingual epithelium in rodent embryos (Liu et al. 2004), and its expression stays constant through the development with only small fluctuations (Bitgood and McMahon 1995, Hall et al. 1999). It was previously reported that disruption of Shh signalling increases papillae number and causes papillae to appear in ordinarily unusual places, such as the intermolar eminences, which is a lingual region of the E13.5-E15 embryo that does not normally contain papillae (Mistretta et al. 2003, Mistretta and Liu 2006). It was also determined that Shh signal disruption causes disturbance of tongue formation (Liu et al. 2004). These actions, however, seem to affect only the fungiform papillae on the anterior part of the tongue, while the circumvallate papilla on the posterior part is not affected (even though Shh is expressed in it) (Mistretta et al. 2003). It was then found that it may be the taste bud rather than the papillae that are developed as a result of expression of Shh (Thirumangalathu et al. 2009 (discussed later)).

Another factor that plays an important role in fungiform papillae formation is the family of bone morphogenic proteins, BMPs. BMPs are a small family of secreted factors belonging to family of large transforming growth factors β (TGFβ) (Kingsley 1994). BMPs 2, 4, and 7 are expressed throughout the epithelium and mesenchyme (Jung et al. 1999), and have been shown to be able to disrupt the formation of fungiform papillae on the tongue (Zhou et al. 2006). This disruption

can be reversed by Noggin, a BMP antagonist. Noggin was found to induce papillae formation (as well as occasional incidence of fused fungiform papillae), a result that was not repeated by another BMP antagonist, follistatin, presumably because follistatin binds BMPs with only a low affinity, differently to Noggin, which binds BMPs with high affinity (Zhou et al. 2006, Balemans and Van Hul 2002). Interaction with Shh has been suggested due to a remarkably precise, one-to-one association of placodal cells expressing BMP4 and Shh (Hall et al. 2003).

Wnt signalling has also been found to play a role in papillae formation: canonical Wnt10b signalling through β-catenin and Lef1 is required for fungiform papillae development (Iwatsuki et al. 2007). In Wnt10b knockouts (same as in Lef1 and β-catenin knockouts), large losses of fungiform papillae are observed, an effect that is not seen in circumvallate papilla (Iwatsuki et al. 2007, Liu et al. 2007). Furthermore, *Lef1* null postnatal tongue contains much larger population of filiform papillae, presumably at the expense of fungiform papillae (Iwatsuki et al. 2007). Recently, yet another Wnt factor was found to influence the development of these papillae. Wnt5a (whose levels are distinctly higher in anterior tongue compared to other lingual regions (Liu et al. 2009)) has been found to affect fungiform papillae as well, on top of altering the size of the tongue itself: Wnt5a knockout mice' tongues were much smaller in size when compared to their wild type counterparts (Liu et al. 2012). While it could be expected that the decreased size of tongue would cause smaller number of papillae to develop, this was in fact not the case: the mutants retained the same number of fungiform papillae on their tongues as were observed in wild types, albeit the density and positioning of these papillae was largely altered.

Since downregulation of Shh signalling (in cultured tongue explants) has been shown to upregulate Wnt/β-catenin signalling, the interaction between Shh and Wnt pathways is deemed to be important in fungiform papillae formation (Iwatsuki et al. 2007, Mistretta and Liu 2006). Furthermore, Shh signalling (as previously mentioned) is important in maintaining spatial pattern of fungiform papillae. One would therefore expect that there would be an interaction between

Wnt5a and Shh, for instance, however, the precise relationship is yet to be determined.

Wnt signalling is also important for maintaining the expression of *Sox2* (Okubo et al. 2006). This is important during the development of the fungiform papillae as Sox2 plays a role in differentiation of papillae to fungiform and filiform. Reduced expression of *Sox2* caused a large number of filiform papillae to develop, whereas the number of fungiform papillae was reduced (Okubo et al. 2006).

Finally, the epidermal growth factor (EGF), at least in tongue cultures, caused the formation of fewer fungiform papillae in a dose-dependent manner when added to the medium (Liu et al. 2008), and its interaction with Shh while influencing the number of fungiform papillae has been suggested (Krimm 2007). However, *in vivo* studies have shown that the epidermal growth factor receptor supports epithelial differentiation and postmitotic neurons (Sun and Oakley 2002, Xian and Zhou 2000). *Egfr* (EGF receptor) null mice were found to have deficiencies in the anterior gustatory epithelium (Sun and Oakley 2002).

There are, however, other factors that play a role in tongue and papillae development, although their roles have not been determined to a large extent. For example, the FGF (Fibroblast growth factor) and Notch pathways have been suggested to influence the formation of these organs as well. FGF8 has been found to be expressed in the lingual epithelium, but this expression subsides when the taste placodes start to appear (Jung et al. 1999), suggesting that perhaps FGF8 influences tongue formation but has a limited role in taste placode development. Notch expression is not detected in the tongue until after the expression of Wnt, Shh and BMP have entered within the papillae (Seta et al. 2003). It is deemed that Notch plays a role in this process as well, perhaps later in morphogenesis of the papillae, but the precise function has not been determined.

Whether or not the initial development of these papillae happens prior and independently to the actual innervation has been debated over many years (Mbiene and Mistretta 1997). The pattern that these papillae form on the tongue therefore presents a predictable and discrete target for the lingual afferents from

the trigeminal and the geniculate ganglia and would thus suggest that innervation is perhaps not necessary to initiate their formation. How is the targeting into the neural/taste buds facilitated? When does it happen, and does it need to be initiated/completed before the taste buds start to form? What are the mechanisms that influence and guide the lingual afferents as they descend from the geniculate ganglion to the fungiform papillae located on the dorsal surface of the anterior tongue? To answer these questions, one needs to look at the development of the taste buds themselves in detail.

Development of taste buds

It has been widely accepted that there are essentially three main stages in development of the taste buds on the tongue: the initial period establishing the localization and competence of the taste papillae, followed by sensitive periods when innervation is required for papillae maturation and taste bud formation, and finally a period of proliferation and differentiation of taste receptor cells (Oakley and Witt 2004). The entire population of taste bud cells has been calculated to develop from around 7-13 cells. Once the taste buds are formed, their population is constantly renewed through adult life from a proliferative progenitor pool within the papillary epithelium (Stone et al. 2002, Okubo et al. 2008, Thirumangalathu et al. 2009). Initially the taste buds, embedded within the papillae on the tongue and soft palate in their final form, arise from oral epithelium. The first instances of taste bud progenitors have been detected at about E13.5 mice, using an intermediate filament cytokeratin 8 (Mbiene and Roberts 2003), however, much of the taste bud development and induction is still shrouded in mystery. It has been determined that the development of the taste buds lasts throughout much of the progression of the embryonic stages and that the last part of the taste buds to be developed are the taste pores that only appear during postnatal development (Mistretta et al. 1997, Stone et al. 2002).

Many of the factors that influence the development of fungiform papillae (as discussed above), are also thought to influence the development of taste buds. Factors such as Shh, Bmp and Wnt/β-catenin influence the taste bud progenitors directly (Thirumangalathu et al. 2009). These influence the taste bud until it takes its final form which consists of a heterogeneous population of about a hundred cells, each one belonging to three different cell types as described previously: Type I, II and III.

Despite the early initial embryonic development, taste buds are only differentiated and functional well after the tongue is innervated late in embryonic stages (Thirumangalathu et al. 2009), and they begin to express taste cell markers such as α–gustducin, Shh and Troma-I around birth (Krimm and Barlow 2008). On the contrary to taste placodes and taste papillae, lingual innervation is very important for taste bud maturation as the taste buds appear to differentiate only when gustatory innervation is present (Oakley and Witt 2004). Innervation is also important for taste bud size as it is correlated with the number of geniculate ganglion neurons that innervate it (Krimm and Hill 1998). Normally, adult mouse taste buds are innervated by about 2-7 geniculate neurons (Zaidi and Whitehead 2006).

Around birth, an aggregate of immature cells is present in the apex of each papilla. The timing is not precisely known, but sometime during the first postnatal week, these cells differentiate into a mature taste bud (Krimm and Barlow 2008). This would suggest that taste buds are not fully differentiated at birth, which has been found in studies previously (Oakley et al. 1991, Mistretta and Liu 2006). This is interesting, because newborn mice suckle nearly immediately after birth, which means that they likely posses a mechanism that would enable them to taste. It is likely, therefore, that the role of taste buds on the tongue is taken over by those present on the soft palate, as they have been found to be differentiated prior to birth (Harada et al. 1997, Harada et al. 2000, Sollars and Hill 2005). However, they may not possess a full complement of tasting ability as it is the sweet taste that is the most crucial during the first stages of postnatal development due to suckling.

Because taste buds are expressed solely within taste papillae one would expect that the factors that influence the development of these would also influence the development of the taste buds. Thirumangalathu et al. in 2009 showed that indeed Shh-expressing placodes were in fact taste bud progenitors, and could lead to differentiation of two different taste cell types as well as intragemmal basal and edge cells in adult mice.

Developing taste buds: importance of innervation

In the past there has been an extensive discussion about whether taste buds are able to develop without innervation. Many experiments showed that innervation is required for most taste buds to develop, with a small proportion of some fungiform taste buds being an exception (Fritzch et al. 1997). Experiments that surgically disrupted the innervation caused lack of taste bud development (Hosley et al. 1987, Nagato et al. 1995), as did depletion of BDNF-dependent innervation (Zhang et al. 1997, Nosrat et al. 1997, Mistretta et al. 1999) and TrkB-dependent innervation (Fritzch et al. 1997,) and other studies (Fan et al. 2004, Ringstedt et al. 1999, Krimm et al. 2001). All of these pointed to the fact that without gustatory innervation, only a very small proportion of taste buds develop. Whether the development of taste buds was nerve-dependent and the lack of innervation caused them to deveop and then recede or not develop at all was not clear.

Recently, experiments done by Ito et al. (2010) using Troma-I (cytokeratin-8) staining showed that the neural buds formed before the innervation reached them, thus concluding that innervation was not essential for initial taste bud formation, contrary to the neural induction model. According to the new model, formation of the fungiform placodes is independent of innervation and happens prior to it; however, the synaptogenesis stage, which follows the formation, does require functional connections, thus defining a temporal point in development that signals neuronal dependence of neural buds (Ito et al. 2010).

When the gustatory nerves are severed the taste buds degenerate and only regenerate if the innervation is restored (Cheal and Oakley 1977). Although even after re-innervation some morphological changes remain in place (St. John et al. 1995). This happens in adult animals; in developing gustatory system the situation is somewhat different. If the chorda tympani is sectioned in early postnatal animals, the regeneration period does not occur at all, thus causing a permanent loss of fungiform papillae and taste buds (Sollars et al. 2002). Why this is the case is not entirely clear, but it is possible that because the sectioning happens in early postnatal life of the animal, the fungiform papillae and their taste buds are not yet under maintenance control of NT4, and are thus more susceptible to a loss of innervation than in the adulthood. This could then mean then loss of innervation causes morphological change, which prevents re-innervation and thus also the rescue of these morphological effects. Furthermore, these changes are dependent on the location of the taste buds on the tongue itself; the taste buds present in the very tip of the tongue are less susceptible to the nerve sectioning than taste buds present in the middle of the tongue (Guagliardo et al. 2007).

Innervation and targeting

As mentioned previously, the fungiform papillae are targeted by the chorda tympani fibers, while the surrounding epithelia are innervated by the somatosensory lingual branch of the trigeminal nerve (Ringstedt et al. 1999). In mice, the chorda tympani is the first nerve entering the tongue. Only after it reaches the tongue it is followed by the lingual fibers (Whitehead and Kachele 1994).

Upon development of the papillae, the initial targeting takes place by E14.5 in mouse when the chorda tympani fibers enter the caudal lateral point of the tongue. From there they grow rostrally along the base of the tongue to branch out underneath the epithelial layer and innervate the papillae on most of the anterior dorsal tongue surface (Lopez and Krimm 2006). During innervation, nerves follow

very precise and spatially restrictive pathways, which suggests that guidance is provided by molecular signals in the environment (Mbiene and Mistretta 1997, Rochlin et al. 2000). The fibers travel from the ganglia into the tongue as a single bundle. When they penetrate the epithelial surface near the papillae, they defasciculate, branch abundantly, and form a neural bud (Lopez and Krimm 2006).

Each taste bud is innervated by 2-7 geniculate ganglion cells (Zaidi and Whitehead 2006). By injecting taste buds in different regions of the tongue with colour markers Zaidi and Whitehead also showed that there was no topographic map that would represent the taste buds from the tongue in the geniculate ganglion, even though there is a very stereotypical organization of the fungiform papillae (and hence taste buds) on the tongue. When this is compared to the topographical taste map in the geniculate ganglion as showed by Barretto (2012), one can postulate that despite there being a topographical organization in the geniculate ganglion, this does not necessarily reflect the morphological organization of the taste buds. This means that the functionality of each taste bud is what organizes this map, rather than its localization on the tongue.

The gustatory afferents are first seen in the gustatory epithelium on the tongue just before the initial formation of the fungiform papillae (Farbman and Mbiene 1991, Whitehead and Kachele 1994), and once they innervate these, the innervation is essential for the neural bud maintenance and growth (Nagato et al. 1995). Papillae and taste buds provide discrete and predictable targets for innervating lingual afferents, and each one is innervated by a specific number of neurons (Krimm and Hill 1998, Zaidi and Whitehead 2006). However, some errors in targeting do occur. During the initial stages of innervation, some fungiform papillae do not get innervated, while there are also regions on the tongue that receive lingual innervation despite the lack of presence of any of the fungiform papillae. There is, however, a post-targeting refinement period between E14.5 and E18.5 that corrects most of these mistakes so that by the end of embryonic development more than 98% of fungiform papillae are innervated correctly and less than 5% of fibers appear in areas containing no fungiform papillae (Lopez and Krimm 2006).

The targeting into the papillae itself is done by a mechanism described previously as "brush-like endings" with filopodia (Mbiene et al. 2004), which seem to sample the local environment and help detect any guidance cues.

Factors that influence targeting of the geniculate axons into the tongue

The guidance of the axons from the gustatory ganglia is stereotyped and appears to follow predetermined pathways. It was thus assumed that the guidance is facilitated by molecular cues in the environment, and in order to show that this was the case, studies have set out to prove the mechanisms that would provide the paths that the axons would follow. This thesis focuses on the innervation into the tongue, although the geniculate ganglion has been shown to project into the palate as well.

One of the first factors found to have a role in this guidance was Semaphorin 3A. Semaphorin 3A was found to be expressed throughout the developing tongue (Giger et al. 1996), and its expression was found to form a gradient: it increases from lateral to medial part of the tongue surface and has been shown to prevent untimely growth of both trigeminal and gustatory fibers into the tongue (Rochlin and Farbman 1998, Rochlin et al. 2000). Together with this, Semaphorin 3A was shown to prevent early penetration of the epithelial surface by the geniculate fibers (Vilbig et al. 2004).

Some of the most important factors that are involved in the guidance are neurotrophins. Culture studies showed that geniculate ganglion axons are attracted towards beads that contain BDNF (Hoshino et al. 2010). Initial studies determined that the first outgrowth of axons from the gustatory ganglia required addition of neurotrophins *in vitro* such as NT4 and BDNF that exerted their influence on the outgrowth of geniculate axons at various stages of development. In these experiments, Rochlin et al. (2000) showed that both of these neurotrophins promote axon outgrowth at early stages (E12.5), however, this influence is decreased, at least in the case of NT4, by E14.5 (Rochlin et al. 2000).

Examining the expression of neurotrophins in the gustatory system showed that most of those found involved were expressed in the desired areas. Furthermore, the expression of several neurotrophins drastically changed throughout the mouse gustatory embryonic development, reflecting the need for changes of guidance cues during targeting.

BDNF mRNA was found to be highly expressed in cells of the developing gustatory epithelium, specifically in locations of future fungiform papillae, as well as in adult taste buds later in development (Nosrat et al. 1996, Huang and Krimm 2010). The expression occurs independently of innervation (Nosrat et al. 2001), and continues to be expressed during development and through adulthood (Ganchrow et al. 2003, Yee et al. 2003), so the presence of BDNF may help the fibers to target the gustatory papillae and distinguish them from non-gustatory papillae (such as the filliform papillae). In the geniculate ganglion, the expression of BDNF mRNA was found to be high at the initial stages of gustatory development, and even increase during the later embryonic stages.

The expression of NT4 mRNA was shown to be primarily robust, but to then decrease steadily throughout development: between E12.5 and E14.5 its expression decreases in all areas of expression (Huang and Krimm 2010), upon which it stabilizes. As mentioned previously, Rochlin et al. (2000) showed that while at E14.5 the expression of NT4 mRNA decreases, so does the responsiveness of geniculate ganglion axons to this neurotrophin *in vitro*.

On the contrary to the expression areas of BDNF and NT4, the expression of NT3 was found to be located primarily to the somatosensory epithelium. This corresponds with its involvement in guidance of the gustatory tracts as well, however, NT3 has been shown to principally affect the somatosensory fibers targeting into the foliate papillae and the base areas of fungiform papillae (Oakley and Witt 2004).

Involvement of BDNF in geniculate fibers' guidance into the gustatory areas, mainly on the tongue, has been widely studied. The disruption of normal BDNF expression by overexpression in vivo prevented chorda tympani innervation into the

fungiform papillae (Ringstedt et al. 1999, Krimm et al. 2001, Lopez and Krimm 2006). Overexpressing BDNF in non-gustatory areas of the tongue (under the control of keratin 14 promoter) was found to disrupt targeting of the lingual afferents to the fungiform papillae (Krimm et al. 2001, Lopez and Krimm 2006), where instead of the fungiform papillae the fibers were targeting all the other structures on the tongue, mostly the filliform papillae. The same effect was found when overexpressing NT4 (Lopez and Krimm 2006). This was true despite the fact than in both of these cases the number of geniculate ganglia was in fact increased by about 60%.

The claim that BDNF was required for successful targeting of the afferents to the fungiform papillae was further supported by experiments done on *Bdnf* (as well as *Nt4*) null mice. Ma et al. (2009) showed that mice lacking BDNF expression had deficient targeting of the chorda tympani branches into the fungiform papillae (the branches did not reach their targets), although the branching of this nerve in the epithelial parts of the tongue was largely increased. The reason for this might be that because the *Bdnf* gene recombination occurs in the tongue tip earlier than in the rest of the lingual epithelium (E13.5 and E14.5, respectively), the targeting of the branches into the tongue itself happened too early to be affected by the gene recombination (Ma et al. 2009). Although a possibility remains where BDNF does direct targeting and its absence would cause fibers to augment their search for targets thus increasing the amount of branching. Either possibility explains why there was an increased amount of branching in the epithelial part of the tongue, but none in the fungiform papillae themselves. It also suggests a critical period during which BDNF is able to influence targeting: the recombination must occur by E13.5 for targeting to be disrupted. Eventually, at E18.5, some of the fungiform papillae were found to be innervated despite the lack of BDNF expression (Ma et al.2009). This could be explained by hyperinnervation that was seen in the epithelial layer around the taste buds: since the initial innervation does not target the fungiform papillae, there is an increased level of branching. This increases until about E17.5

when few of the branches penetrate the epithelium and form connections with the papillae.

Interestingly, the same study showed that *Nt4* null mice did not affect the chorda tympani branching and innervation to the same extent that *Bdnf* null did (although a small loss of innervated fungiform papilla was observed, which would be compliant with the loss of neurons seen in these animals as will be discussed later), even though mice overexpressing this neurotrophin did (Lopez and Krimm 2006, Ma et al. 2009). Recent studies by Runge et al. 2012 showed, however, that NT4 is perfectly capable of attracting neurites from the geniculate ganglia, and perhaps even more so than BDNF. These experiments, together with the ones by Lopez and Krimm 2006 and Ma et al. 2009 suggest that despite being able to, NT4 is not involved in chemoattraction of these neurons to the fungiform papillae. Why this might be the case will be discussed later. When examining the double *Bdnf* and *Nt4* null mice, Ma et al. 2009 found that while branching patterns seemed normal or increased in either *Nt4* null or *Bdnf* null mice, it was nearly absent in the double mutant mice. This supports the idea that NT4 has a role in branching of the chorda tympani fibers rather than in targeting of these fibers to the gustatory papillae.

One of the factors that may play an important role in the guidance of the geniculate ganglion axons during targeting is that each sensory nerve arising from the geniculate ganglion expresses a unique fingerprint of neurotrophins and neurotrophin receptor genes (Farbman et al. 2004). This, together with the variable expression of the BDNF and NT4 may finally determine the path which an axon from the geniculate ganglion would finally take to reach its destination.

Furthermore, it was found that loss of each of these two neurotrophins causes loss of both the taste buds on the tongue and the gustatory papillae (Mistretta et al. 1999, Nosrat et al. 1997, Oakley 1998, Liebl et al. 1999), which could be seen as a result of the loss of innervation if the taste buds indeed do require neuronal support for their survival.

1.2.4. Influence of neurotrophins on geniculate ganglion neuronal survival

The role of BDNF and NT4

While in some systems neurotrophins have been found to have a target derived role, where they are produced in the targets of growing axons and thus regulate the number of surviving neurons, neurotrophins can also be found along the axonal projections of sensory neurons regulating both survival as well as the pathways these neurons take. Neurotrophins are, however, also produced in and near sensory ganglia influencing cell cycle kinetics and differentiation. For example, the trigeminal ganglion neuron recursors do not survive in absence of NT3 (Elshamy and Ernfors 1996), and the dorsal root ganglion neurons require NT3 to regulate neuron number during development (Farinas et al. 1996). The geniculate ganglion produces BDNF (Schecterson and Bothwell 1992), implying the possible survival-dependency of these neurons on BDNF. The nucleus of the solitary tract, the primary target of the geniculate neurons, also produces BDNF (Acheson et al. 1995). NT4 is also expressed in the geniculate ganglion (Cho and Farbman 1999, Farbman et al. 2004). BDNF and NT4 are therefore the prime candidates to influence cell cycle dynamics as well as neuronal differentiation. While these two neurotrophins are required for successful guidance of geniculate ganglion afferents to the tongue (discussed above) the survival of these neurons is not target derived; at least during the initial stages of development.

Experiments done in culture showed that only BDNF and NT4 are able to support the population of geniculate ganglion neurons as a large amount of neurons survived with these neurotrophins in culture, which was not the case when NGF and NT3 were used: only about 10% of neurons survived in these instances (Al-Hadlaq et al. 2003). Examining mice with knockouts of either BDNF or NT4 it was found that mice lose about half of the neuronal population in the geniculate ganglion. Importantly, however, this happens at different times of development:

Nt4$^{-/-}$ mice lose their neurons starting at E12.5, which is well before the afferents start targeting the neural buds. On the other hand *Bdnf*$^{-/-}$ mice start losing first geniculate ganglion at a later stage, between E12.5 and E14.5 (Conover et al. 1995, Liu et al. 1995, Patel and Krimm 2010a). *Nt4*$^{-/-}$ mice then keep losing neurons until about E16.5, when this loss of neurons steadies itself and the neuronal number stops changing. *Bdnf*$^{-/-}$ mice, however, keep losing the geniculate ganglion neurons through E18.5. Thus, both NT4 and BDNF are involved in regulating the numbers of neurons in the geniculate ganglion where NT4 regulation both begins and ends earlier than the influence of BDNF (Conover et al. 1995, Liu et al. 1995, Erickson et al. 1996, Liebl et al. 1999, Patel and Krimm 2010a). The nodose/petrosal ganglion is also influenced by the lack of these neurotrophins. *Bdnf*$^{-/-}$ mice were found to have about 50% decrease in the population of the nodose-petrosal ganglion, whereas *Nt4* null mice showed nearly 60% decrease of the population in this ganglion during development (Ernfors et al. 1994, Jones et al. 1994).

The losses shown in the single-mutation geniculate ganglia are additive. Examinations of double *Bdnf/Nt4* null mice found that the loss of the neurons equals to ninety percent of the neuronal population of the ganglion, with the same numbers showing in the *Trkb* null mice, discussed below (Conover et al. 1995, Fritzsch et al. 1997). Because of this it may be that BDNF and NT4-dependent neurons represent two different neuronal populations.

The effect to which the neurons in the geniculate ganglion are dependent on each neurotrophin has been a point of several studies. In 2010, Patel et al. (b) labelled fibers from the soft palate and the tongue with DiI and counted neurons in the geniculate ganglion in *Bdnf*$^{-/-}$, *Nt4*$^{-/-}$ and *Bdnf*$^{-/-}$/*Nt4*$^{-/-}$ mice. They found that the neurons in this ganglion were equally dependent on both neurotrophins as neurons innervating the soft palate lost 30% and 40% of neurons in *Bdnf*$^{-/-}$ and *Nt4*$^{-/-}$ mice, respectively, whereas loss of 50% and 60% was seen in neurons innervating the tongue. However, only about 50% of the neurons in the geniculate ganglia were accounted for in this study (the authors counted only 500 neurons in the ganglia). Some of these could be speculated to be of somatosensory nature, innervating the

vallate papillae posterior to the soft palate or perhaps not be gustatory at all. Therefore the neurotrophin dependency of neurons in this ganglion has not yet been entirely determined.

The role of NT3 in geniculate ganglion neuron survival

NT3 has also been found to have influence on the number of geniculate ganglion neurons. NT3 is expressed in the lingual epithelium surrounding the taste buds, but not in the taste buds themselves (Nosrat et al. 2004). *Nt3* null mice have been found to lose about 35-45% of the geniculate and nodose/petrosal neurons (Farinas et al. 1994, Liebl et al. 1997). These mutants did not appear to influence the morphology of fungiform papillae and taste buds on the tongue to a great extent, unless they were concomitant with *Bdnf* null mutants: *Bdnf* and *Nt3* null mice lost more fungiform papillae than *Bdnf* null mice (*Nt3* null mice did not lose any), (Nosrat 1997, Nosrat 2004). This would suggest that NT3 has a more supportive than direct role, and that it perhaps supports the maintenance of fungiform papillae in the absence of BDNF. It could also mean that NT3 has a nerve independent influence on these papillae.

Facilitating the loss of geniculate ganglion neurons

How is the loss of geniculate ganglion neurons facilitated in the absence of BDNF? Once it was established that *Bdnf* null mice lose neurons in the geniculate, trigeminal and nodose/petrosal ganglion, several studies examined how these neurons were lost.

One of the primary candidate mechanisms facilitating this loss is apoptosis. Hellard et al. (2004) showed that deleting the proapototic gene *Bax* rescued neuronal apoptosis in the developing trigeminal and nodose-petrosal ganglia and therefore showed that BDNF signalling was important for suppressing Bax-mediated cell death in the ganglia. The same effect was shown in the geniculate ganglion by

Patel and Krimm (2010a) who showed that in *Bax*$^{-/-}$ mice, *Bdnf* null mutation had no effect on the numbers of geniculate ganglion neurons. *Bdnf* null mice also contained more neuronal cells with activated caspase-3. Therefore the role of BDNF in regulating the neuronal numbers in geniculate and other ganglia in the periphery is by preventing cell death (by preventing caspase-3 activation).

NT4, however, functions differently. Although its null mutation has been also found to cause loss of about half of the neurons in geniculate ganglion (as mentioned above), the mechanism via which it does so is much less clear. Patel and Krimm (2012) showed that the regulation of cell death happens via mechanism that is independent of caspase-3 activation. This suggests that the way BDNF and NT4 influence the geniculate ganglion neuronal population is different, however, the actual mechanism of NT4 influence is yet to be determined.

Expressing BDNF under the control of Nestin promoter (which directs the expression to neuroepithelial stem cells of the central nervous system as well as peripheral nervous system and to developing muscle (Ringstedt et al. 1999, Lendahl et al. 1990)) showed that the innervation patterns were severely affected in these mice and that gustatory axons failed to innervate the targeted taste buds and were halted in the tongue muscle. This initially showed that BDNF was important for afferent targeting into the taste buds. Subsequent experiments using a keratin-14 promoter (to drive the expression in the epithelium layers such as the skin and tongue (LeMaster et al. 1999)) showed that overexpressing these neurotrophins had influence on not only the neuronal targeting of the lingual afferents into the tongue, but on the number of surviving geniculate ganglion neurons as well. BDNF-overexpressing ganglia had a 93% increase in the neuronal population, whereas the NT4-overexpressing ones had a 140% increase. At the same time, however, the number of fungiform papillae as well as taste buds was decreased in both instances. This would indicate that while the overexpression was directed into non-gustatory epithelia, the fibers were able to target areas that contained expression of these neurotrophins, thus innervating areas that previously lacked innervation, such as the filiform papillae (Krimm et al. 2001).

The role of TrkB on maintenance of geniculate ganglion neurons

As mentioned above, both BDNF and NT4 signal through the same receptor, TrkB. The geniculate ganglion neurons express TrkB during the early stages of development (Yamout 2005), although some of these neurons change the expression of TrkB for one of other neurotrophins receptor such as TrkA, TrkC as well as p75NTR (Cho and Farbman 1999). *Trkb* mRNA is highly expressed in the geniculate ganglion throughout the development and is much higher than either BDNF or NT4 mRNA (Yamout 2005), however, in the tongue and soft palate the TrkB mRNA levels are very low (Huang and Krimm 2010).

Geniculate ganglion neurons are lost in *Trkb* null mice starting at E12.5, and by E13.5 this loss exceeds the loss of neurons in either *Bdnf* or *Nt4* null mice (Fritzsch 1997). This, together with the fact that both *Bdnf/Nt4* null mice and *Trkb* null mice lose about the same amount of neurons suggests that both of these neurotrophins signal through this receptor to influence neuron number (Erickson 1996).

$Trkb^{SHC}$, $Trkb^{PLC\gamma}$ and Trk^{D} mice can be used for studying TrkB signalling pathways

To dissect the roles of TrkB signalling pathways on the gustatory system, mice carrying mutations in each of the two main adaptor sites, TrkB/Shc and TrkB/PLCγ, were used to identify which aforementioned characteristics of the mouse gustatory system would be affected. Mice with these mutations were previously generated, in particular the $TrkB^{SHC}$ (a Y-F mutation present in the TrkB/Shc adaptor site), $TrkB^{PLC}$ (a Y-F mutation present in the TrkB/PLCγ adaptor site) and $TrkB^{D}$ (a double mutant containing both aforementioned mutations). Due to the genetic strategy used to generate the TrkB/PLCγ mutation and the double mutant (cDNA knock-in) a control line was generated with a similar strategy ($TrkB^{WT}$) and used in experiments involving the above lines (Minichiello et al. 2002).

These mouse lines have been used in other experiments previously. In particular, Minichiello et al. 98 showed that mutation at the TrkB/Shc adaptor site causes mild reduction in vestibular ganglia neurons, and a partial loss in nodose petrosal ganglion neurons (Minichiello et al. 1998, Fan et al. 2000), although the growth of fibers from the vestibular ganglion as well as target innervation was lost in mice with this mutation (Postigo et al. 2002). Importantly, D-hair receptors showed 95% loss in these mutants, which was shown to be identical to *Nt4* null mutants. These experiments also showed that the BDNF/TrkB neuronal differentiation was not affected in TrkB/Shc mutant mice.

Experiments with mice bearing mutation in the TrkB/PLCγ site showed target innervation defects of the vestibular sensory neurons; however, no loss of neurons was shown (Sciaretta et al., 2010, Medina et al. 2004). TrkB/PLCγ docking site was also shown to be important for TrkB-mediated hippocampal synaptic plasticity (Minichiello et al. 2002). The double mutation was shown to have a crucial influence on the vestibular sensory neurons and growth toward the sensory epithelia as it resulted in a loss that was virtually indistinguishable from *Trkb* null mice (Sciaretta et al. 2010).

Both BDNF and NT4 have been previously shown to play an important role in regulating the geniculate ganglion neuronal survival as the null mutations of these neurotrophins caused losses of large numbers of these neurons during embryonic development. They were also shown to play a role in targeting of the geniculate ganglion afferents into the tongue. Because double null mutations of these neurotrophins mirrored the effects seen in experiments with *Trkb* null mice, it was suggested that the survival and guidance function mediated by BDNF and NT4 signalling would be through the TrkB receptor (Fritzsch et al. 1997, Conover et al. 1995, Liu et al. 1995). Consequently, this would be mediated by the two major adaptor sites (TrkB/Shc and TrkB/PLCγ1).

1.3 Aims of experiments

The aim of this project is to determine which TrkB intracellular signalling pathways are required for specific aspects of gustatory development. In particular,

- which of the signalling pathways downstream of two major docking sites (TrkB/Shc and/or TrkB/PLCγ) are required for survival of the geniculate ganglion neurons
- which of these signalling pathways are involved in innervation of neural and taste buds during gustatory development
- whether the signalling via TrkB docking sites is involved in morphology of taste buds in the developing taste system
- by comparing to previous data, to determine which TrkB ligand is involved in the above processes

The goal of these studies is to expand the understanding of how BDNF and/or NT4, by signalling via TrkB, control development and maturation of rodent gustatory system and dissect possible differences in signalling pathways between developing and adult systems. The results provide requisite knowledge for studies that will focus on functionality as well as plasticity of both neurons and taste buds in this system.

2. MATERIALS AND METHODS

2.1 Chemicals and consumables

2.1.1 Chemicals

All chemicals were purchased from Sigma Aldrich (Sigma-Aldrich Ltd, Gillingham, UK and Sigma-Aldrich Chemie GmbH, Munich, Germany) or VWR (VWR International, Lutterworth, UK and VWR International GmbH, Darmstadt, Germany) in molecular biology grade.

Chemicals

$(HOCH_2)_3CNH_2$ (Tris buffer)

$C_{10}H_{16}N_2O_8$ (EDTA, ethylenediaminetetraacetic acid)

$C_{12}H_{22}O_{11}$ (sucrose)

C_2H_6O (ethanol, EtOH)

$C_3H_8O_3$ (glycerol)

$C_6H_5CH_2O_2CC_6H_5$ (benzyl benzoate)

$C_6H_5CH_2OH$ (benzyl alcohol)

CH_3COOH (glacial acetic acid)

CH_3COONa (sodium acetate)

CH_3OH (methanol)

H_3BO_3 (boric acid)

HCl (hydrochloric acid)

KCl (potassium chloride)

Na_2HPO_4 (disodium phosphate)

$NaC_{12}H_{25}SO_4$ (SDS, sodium dodecyl sulphate)

NaCl (sodium chloride)

NaH_2PO_4 (monosodium phosphate)

NaN_3 (sodium azide)

NH_2CH_2COOH (glycine)

NH_4Cl (ammonium chloride)

NH_4OH (ammonium hydroxide)

$OH\ (CH_2O)_nH$ (n = 8 - 100)(PFA, Paraformaldehyde)

2.1.2 Consumables

0.2ml 8-strip PCR tubes (I1402-3508, Starlab)

1kb ladder (250µg) (15615-016, Invitrogen)

3M Comply Indicator Tape Class 1 (3M)

Agarose (A9539-500G, Sigma Aldrich)

Agarose, low gelling temperature (A9414-500G, Sigma Aldrich)

Calf serum (C8056-100ML, Sigma Aldrich)

Coverslips (24x55mm, 630-1596, VWR)

Cresyl Violet Acetate (C5042-10G, Sigma Aldrich)

DAB tablets (D5905-50tab, Sigma Aldrich)

DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, D-3911, Invitrogen)

DMSO (472301-100ML, Sigma Aldrich)

dNTPs; 100mM dNTP Set (4x25umol) (Invitrogen)

DPX Mountant for histology (06522-100ML, Sigma Aldrich)

Eosin (Accustain Eosin Y Solution aqueous, HT110216, Sigma Aldrich)

Eppendorf Safe-Lock microcentrifuge tubes, 1.5ml (T9661-1000EA, Sigma Aldrich)

Ethidium Bromide (E1510-10ML, Sigma Aldrich)

GO buffer (M791A, Promega)

GO Taq polymerase (M3001, Promega)

Hematoxylin (Accustain Harris Haematoxylin solution, HHS16, Sigma Aldrich)

Injection needles (Becton Dickinson Plastipak)

Liquid scintillation vials, 20ml (Z253081-1PAK, Sigma Aldrich)

Normal goat serum (S-1000, Vector labs)

NUNC CryoTubes 1.8ml (177280, Thermo Scientific)

OCT (Mounting medium for cryotomy, 361603E, VWR)

OrangeG (O3756-100G, Sigma Aldrich)

Paraffin wax (A6330-1CS, Sigma Aldrich)

Parafilm (PH-LF-PM996-EA, Pechiney)

Peel-A-Way Disposable Embedding Molds (186646 A, Polysciences)

Petri dish, 50mm (122, Sterilin)

Phosphate Buffered Saline tablets (x100) (P4417-100TAB, Sigma Aldrich)

Pipette tips (filter) (Greiner Bio-one)

Primers (Sigma Aldrich)

Proteinase K (solution) recombinant, PCR Grade, 25ml (03115844001, Roche)

Stripettes; 5ml, 10ml, 25ml (Corning Incorporated)

SuperFrost Plus Slides, white (631-0108, VWR (SciQuest))

Syringes, 1ml, 5ml (Becton Dickinson Plastipak)

Taq polymerase (Proteomics Core Facility at EMBL-Heidelberg)

Thimerosal (T5125-10G, Sigma Aldrich)

Triton X-100 (T9284-100ML, Sigma Aldrich)

Tween 20 (170-6531 BioRad)

Vectashield mounting media for fluorescence (H-1000, Vector Labs)

Whatman qualitative filter paper, Grade4, circles, diameter 90mm (Z240540-1PAK, Sigma Aldrich)

Antibodies

P2X3

Rabbit-α-P2X3 (polyclonal antibody), Millipore (AB5895)

Troma-I

Rat α-cytokeratin 8 (monoclonal antibody), Developmental Studies Hybridoma bank, University of Iowa

Tuj1

Mouse α-neuron-secific beta-III Tubulin (monoclonal antibody), R&D Systems (MAB1195)

2.1.3 Buffers

All buffers were prepared with ultrafiltered water unless stated otherwise. Water was purified using the "Milli-Q-water purification system" from Millipore (Millipore Ltd, Watford, UK). In alphabetical order.

Acidic alcohol
70% EtOH + 0.25% HCl

BABB
33.3% benzyl alcohol, 66.7% benzyl benzoate

Bluing solution
0.3% Ammonium hydroxide in dH_2O

Cresyl violet buffer
25mM Sodium Acetate, 3% Glacial acid in dH_2O

Cresyl violet stock solution
2% (w/v) cresyl violet in dH_20, stir o/n

Cresyl violet working solution
1% cresyl violet stock solution in CV buffer

Cryoprotection buffer
30% sucrose in dH_2O, 0.04% Sodium Azide

Dario's quenching solution
0.05% NaN_3, 0.1M glycine, 0.1M NH_4Cl, 50mM Tris

Dent's fixative
20% DMSO, 80% Methanol

Gel loading buffer 10X
50% glycerol, 0.2% OrangeG (Sigma, O-1623) in 1xTBE

PB
100mM PB, pH 7.4 (7.74% of 1M Na_2HPO_4, 2.26% of 1M NaH_2PO_4)

PBS
0.01M phosphate buffer, 0.0027M potassium chloride and 0.137M sodium chloride, pH 7.4 (was prepared from Sigma PBS tablets - P4417)

4% PFA
For 1 litre final solution 40g of paraformaldehyde (Sigma, stored at 4°C) were dissolved in 800ml PB buffer (pH 7.4) by heating to 60°C and stirred o/n. The solution was cooled down to room temperature, 200ml PB buffer was added, and the solution was adjusted to pH 7.4 with sodium hydroxide if necessary, and filtered through a 50µm syringe filter into 50ml aliquots. Aliquots were stored at -20 °C. For experiments the required amount was thawed and cooled to 4 °C.

Quenching solution
0.05% NaN_3, 0.1M glycine, 0.1M NH_4Cl, 50mM Tris

Taq 10x buffer
100mM Tris-HCl pH9.0, 500mM KCl, 1% Triton X-100

Tail lysis buffer
100mM Tris, 1mM EDTA, 250mM NaCl, 0.2% SDS, pH 7.5

TB
50mM Tris, pH8.3

10X TBE
890mM Tris, 890mM boric acid, 20mM EDTA, pH 8.3

TBS
50mM Tris, 150mM Nacl, H 7.5

2.2 Animals

Animal handling and maintenance

All animal procedures were in line with the UK legislation (Scientific procedures ACT 1986), the University of Edinburgh ethical review committee policy as well as the Home Office Regulation guidelines. The animals used in these experiments were kept on a mixed genetic background (C57B6/129). Animal handling at the Centre for Neuroregeneration in Edinburgh was performed by Jenni Rennie and Lynn Morrison.

For obtaining embryonic litters, adult mice were sacrificed by cervical dislocation; P0 pups were sacrificed by injections of sodium pentobarbital. All other animals used (all embryos) were culled by placement on ice before continuing a procedure.

Mouse lines used in the experiments

Mouse lines used in these experiments were created previously; we used three different mouse lines that contained a point mutation in either of the two adaptor sites on the TrkB receptor, or in both of these. The $Trkb^{SHC}$ line contains a Y515F point mutation, which disrupts the SHC adaptor site (Minichiello et al. 1998), the $Trkb^{PLC}$ line contains a 816Y-F point mutation that disrupts the PLCγ1 adaptor site and $Trkb^{D}$, a line containing a double point mutation in both aforementioned adaptor sites (Minichiello et al. 2002, Medina et al. 2004). As a control for $Trkb^{PLC}$ and $Trkb^{D}$ we used a wild type knock-in mouse line $Trkb^{WT}$ to control for the genetic

strategy used to generate these two lines, the cDNA knockin (Minichiello et al. 2002). Colonies were maintained by Dr. Jacqui Horn.

Sample sizes

N numbers indicated in the experiments represent the total number of animals per genotype used. Table 1 shows the number of litters all the experimental animals were obtained from.

Genotype	Age	Number of animals	Number of litters
Trkb$^{D/D}$	P0	4	3
	E16.5	3	3
	E14.5	3	2
	E12.5	3	2
Trkb$^{P/P}$	Adult	3	2
	P4	3	1
	P0	5	2
	E16.5	3	2
	E14.5	6	3
	E12.5	2	1
Trkb$^{S/S}$	Adult	3	2
	P4	2	2
	P0	3	1
	E14.5	3	2
	E12.5	3	2
Trkb$^{W/W}$	Adult	3	1
	P4	3	2
	P0	6	3
	E16.5	3	2
	E14.5	3	2
	E12.5	4	1
+/+	Adult	3	3
	P4	4	3
	P0	3	3
	E14.5	3	3
	E12.5	4	2

Table 1: Number of animals and litters used in all experiments

2.3 Genotyping

DNA extraction

Genotyping samples from embryos and young pups were obtained by tail clipping. The samples were immersed in 100µl of tail lysis buffer and 1.5µl of proteinase K and put into a Thermomixer Compact (Eppendorf) o/n at 56°C at 600rpm. In order to deactivate proteinase K the temperature was raised to 84°C and the samples were left for 30 minutes and then spun in an Eppendorf Centrifuge 5417R for 1 minute at 6797rcf (8000rpm). The DNA samples were stored at 4°C before genotyping and then transferred to -20°C for storage.

For cleaner DNA we used the Quiagen DNeasy Blood & Tissue Kit. In this protocol, a tail sample is immersed in 180µl in Quiagen Buffer ATL in a 1.5ml microcentrifuge tube. 20µl of proteinase K is added, and the sample is vortexed before placed into a Thermomixer at 56°C for a few hours to o/n. Then, the samples are vortexed for 15 seconds each, and 200µl of Quiagen Buffer AL is added to the sample, together with 200µl of 100% EtOH. The samples are vortexed, and the mixture is transferred into a DNeasy Mini spin column placed in a 2ml collection tube. The tubes are then spinned at 6797rcf (8000rpm) in an Eppendorf Centrifuge 5417R for 1 minute. Flow through and the collection tubes are discarded, and the spin column is placed in a new 2ml collection tube. 500µl of Quiagen wash Buffer AW1 is added, and the samples are spinned again for 1 minute at 6797rcf (8000rpm). The flow-through and the collection tube is discarded again, and the spin column is placed in another new collection tube. 500µl of Quiagen wash Buffer AW2 is added and spinned for 3 minutes at 20817rcf (14000rpm) to dry the DNeasy membrane. The flow-through and the collection tube are discarded again, and the spin column is placed in a clean 1.5ml microcentrifuge tube. 200µl of Quiagen Buffer AE is pipette onto the sample, which is then incubated for 1 minute at RT before it is spun for 1 minute at 6797rcf (8000rpm).

PCR set-up

PCR reactions were set up in 0.2ml 8-Strip PCR Tubes (Starlab). Unless otherwise stated the PCR reaction was formed out of 24µl of mastermix and 1µl of DNA. The mastermix contained 2.5µl 10x buffer, 2.5µl of 25mM $MgCl_2$, 0.625µl of 10mM dNTPs, 0.25µl Taq polymerase, 0.5µl of each primer and dH_20 to fill up to 25µl (17.125µl if two primers were used). The SHC PCR reaction was run differently: the mastermix contained 5µl of 5x GO buffer, 2.5µl of 25mM $MgCl_2$, 0.625µl of 10mM dNTPs, 0.05µl GO Taq polymerase, 0.5µl of each primer and 14.825µl of H_20. All PCR reactions were run on DNAEngine Peltier Thermal Cycler (Bio-Rad).

PCR conditions

Trkb$^{D/D}$, *Trkb*$^{P/P}$ and *Trkb*$^{W/W}$ PCR

Primers:

LM8 5'- CAG CTT CGG TCA TCA GCA ACG -3'

LM9 5'- GCC CAG CAG GAG ACA GAC -3'

LM10 5'- CTC TTG ATG TGC TGA ACA AAT GTG -3'

Product size:

Wild type band:	370bp
Mutant band:	180bp

Cycling conditions: 94°C 2mins, 94°C 10sec, 63°C 10sec, 72°C 45sec, go to step 2 35 times, 72°C 5min.

Trkb$^{S/S}$ PCR

Primers:

LM8 5'- CAG CTT CGG TCA TCA GCA ACG -3'

LM9 5'- GCC CAG CAG GAG ACA GAC -3'

LM33 5'- GAT GTG GAA TGT GTG CGA GGC C -3'

Product size:

Wild type band (LM8 + LM9):	370 bp
Mutant band (LM9 + LM33):	580 bp

The reactions to detect the mutant and wild type bands were run separately.

Cycling conditions: Wild type band; 94°C 2min, 94°C 15sec, 60°C 15sec, 72°C 45 sec, go to step 2 35 times, 72°C 5min. Mutant band: 94°C 2min, 94°C 15sec, 55°C 15 sec, 72°C 1min, go to step 2 40 times, 72°C 5 min.

Gel electrophoresis

Gels used for genotyping were made from 2% Agarose (Sigma) in 0.5x TBE buffer. Agarose was melted in a microwave (around 2 minutes), then let to cool. 5µl of 10mg/ml ethidium bromide solution (Sigma) was added per 100ml of the gel solution before pouring the gel into plastic trays, sealing the sides with tape and attaching desired combs. 6µl of gel loading buffer was added into each sample, and 17µl of each sample was loaded into individual wells. 1kb DNA ladder was used as a marker. Gels were run at 170V for 30 minutes or until the bands were separated, the bands were then viewed on Uvitec Cambridge Gel Document Reader.

2.4 Geniculate ganglion neuron counts

Paraffin embedding: embryonic tissue

Embryos were collected after timed mating aged E12.5 and E14.5. In both instances the procedure involved was identical. The time-mated female was culled by cervical dislocation and the embryos were dissected and placed into a Petri dish with PBS. Individually, each embryo was isolated, its tail used for genotyping, and after decapitation the head was dropped into ice-cold 4%PFA and left at 4°C o/n in a liquid scintillation vial (20ml, Sigma). The next day the tissues were dehydrated by incubating 1 hour at 4° in each .85% saline, 50%, 70%, 85% and 95% EtOH (all in saline). This was followed by 1 hour incubation in 100% EtOH at RT, followed by incubation in the same solution at 4° o/n. The heads were then cleared 2x45min in xylene at RT, and 1 hour in xylene and paraffin 1:1. After one exchange of paraffin, the tissues were left at 56-58° in paraffin o/n. The next day the paraffin was changed further 3-4 times, after which the samples were placed in moulds (Peel-A-Way Disposable Embedding Molds, Polysciences, USA), orientated so that they would be placed horizontally for transversal sections and let dry o/n. The samples were stored at RT.

Paraffin embedding: P4 tissue

P4 pups were obtained and anesthetised with 0.05ml sodium pentobarbital, after which they were perfused first with 0.5ml PBS followed by 1ml 4% PFA. The animals were decapitated, the skin from the top of the head was removed and the heads were placed in into liquid scintillation vials (20ml, Sigma), containing 4% PFA at 4°C for 3 days. Following this incubation the heads were rinsed in PBS 3 times, 5 minutes each. Each head was then processed in the following way: the nose was cut off up to the eyes, as well as the very back of the head and the top off the skull (in order to expose the brain). The heads were then left in 5% formic acid in PBS and

left for 4 days at room temperature, rocking, with the solution being changed after the first two days. After this incubation, the samples were rinsed 3-4 times with PBS and left at room temperature for 1hour, rocking, followed by an o/n wash in 30% EtOH with rocking. The samples were then dehydrated in 50%, 70% and 95% ethanol, the first two incubations for 2 hours, then 95% o/n. After two hour incubation in 100% EtOH the next day, the samples were placed in xylene, which was changed three times over 1.5 hours. Then the solution was exchanged so that it would contain xylene: paraffin (melted at 60°C) 1:1 and left at 60°C for 1hr. The heads were then placed into melted paraffin and the solution was exchanged first after 1 hour, then after 2 hours and the third change was left o/n. The next day, the samples were placed in moulds, let to dry o/n and stored at RT.

Paraffin sections: preparation for stains

Paraffin sections of embryos aged E12.5 and E14.5 were serially cut at 7µm, whereas the sections of P4 animals were serially cut at 8µm. They were then placed onto slides containing 10% EtOH on 42° for 10 minutes. This stretched the paraffin on the slides making the embryonic sections flatter compared to ones using a different procedure. After ten minutes any excess EtOH was drained from the slides using paper towels, and the sections were left to dry o/n at 42°.

Nissl stain

Nissl stain, which stains stomata of neurons, was used for geniculate ganglion neuron counting in the young embryos (E12.5 and E14.5). For this stain the slides were immersed in xylene 2x for 10 minutes to get rid of any wax on the slides. The sections were then re-hydrated in serial dilutions of EtOH; 2x1 minute in 100% EtOH, then 1 minute each in 95%, 80%, 50% EtOH, followed by 10 seconds wash in PBS. The sections were fixed in 4% PFA for 5 minutes followed by 2x5 minute wash

in PBS. A Cresyl violet working solution was prepared by dilution the Cresyl violet stock solution (2% Cresyl violet in distilled water, see buffers and solutions). The slides were then immersed in this solution for 15 minutes, or until the stain was sufficiently dark. The sections were quickly washed in PBS (about ten seconds) and the dehydrated by incubating in serially diluted EtOH solutions for 1 minute in each 50%, 80%, 95% and 100% EtOH. The samples were then placed in xylene for at least 10 minutes. Mounting was done using DPX; after coverslipping the slides were left to dry under a fume hood for several hours to o/n.

Hematoxylin-Eosin stain

In this method, hematoxylin is used to stain nuclei and nucleoli blue, and eosin which counterstains cytoplasm colour. This method was used as an alternative to the Nissl staining for geniculate ganglion counts and proved to be more effective in identifying the ganglia in the sections. As with Nissl stain, the H&E stain was used on embryos aged E12.5 and E14.5.

Previously sectioned and dried slides were put into xylene for 10 minutes to permeate and clear the sections of wax. The sections were then re-hydrated in serially diluted EtOH solutions: 2 minutes in each 100%, 95%, 70% and 50% EtOH. The sections were then placed into hematoxylin (modified Accustain Harris Haematoxylin solution, Sigma HHS16) for 5 minutes. The solution was previously filtered using a filter paper. The samples were then washed in running tap water for 2 minutes for its basic pH. The slides were then subjected to acidic alcohol (70% EtOH, 0.25% HCl) for 15 seconds in order to remove excess haematoxylin stain and to turn the staining red, and washed again for 2 minutes in running tap water. Bluing solutions was then used for 30 seconds to stop the hematoxylin staining and due to its basic pH turns the sections blue. After another 2 minutes in tap water, the slides were submerged in Eosin (Accustain Eosin Y Solution, aqueous, Sigma HT110216, and 0.5% glacial acidic acid) for 30 seconds to 3 minutes to stain the cytoplasm red. Following the red stain the sections were washed in running tap

water for 30 seconds to a few minutes until a stain of desired colour was achieved. The slides were then washed in 95% EtOH for 30 seconds and 100% EtOH for 2 minutes before they were cleared in xylene for at least ten minutes and mounted in DPX. After coverslipping, the slides were left to dry in a fume hood for a few hours to o/n.

Analysis of the transversal embryonic sections for geniculate ganglion counts.

Pictures were taken using a brightfield microscope (Zeiss Axio Scope.A1) using a 63x/1.4 lens and a Zeiss AxioCam 1Cc1 camera. The images were taken as a mosaic using Axio software.

Picture of the whole geniculate ganglion was taken from each section, and each one was then analyzed using ImageJ free software. The volume of the ganglion on each section was calculated, and neurons were counted every 4^{th} section in all instances, together with volumes of all individual neuronal cells. Summary counts were calculated by adding up neuronal profiles. Total counts were then estimated as the product of the number of profiles per volume of the counted sections multiplied by the total volume of the entire ganglion. The number of neurons in a ganglion was calculated by multiplying the total number of neuronal profiles by a correction factor in order to compensate for the possibility that a single nucleus was present in multiple sections. The correction factor was calculated according to the formula: $N=n\times[T/(T\times D)]$, where N is the estimated total number of neurons, n is the number of nuclear profiles, T is the measured section thickness, and D is the average diameter of the nuclei. This estimate was calculated separately for each ganglion based on the average diameter of neuronal nuclei in the ganglion, which were calculated from area measurements for each nucleus. This approach has been used previously to examine numbers of neurons present in various ganglia (Patel 2010, Agerman et al. 2003, Ericskson 1996, 2001 and others)

2.5 Analysis of tongue innervation

Tissue collection and OCT embedding

Tissue collection: E16.5 and P0

Embryos and pups were collected at either E16.5 or P0. The embedding and cutting procedure was identical for both ages.

Upon collection of the litter, the embryos were placed on ice for 10 minutes. In the case of P0 pups, the animals were injected with sodium pentobarbital (0.05ml per animal) and left for ten minutes at RT. The animals were then perfused with 4%PFA and incubated at 4°C o/n in 4% PFA. The tongues and brains were dissected, washed in PBS and placed o/n to 30% sucrose at 4°C. After the incubation the tissue was embedded in OCT (Mounting medium for cryotomy, VWR). First, plastic moulds (Peel-A-Way Disposable Embedding Molds, Polysciences, USA) were filled with liquid OCT, which was then put on dry ice. When the bottom layer of OCT was frozen solid, the tissue was placed in the middle of the mould, which was then immersed in dry ice so that the moulds would be covered on all sides except for the open top. After a few hours on dry ice the samples were transferred to -80°C.

When the tissue needed to be cut, the samples were transferred from the -80°C to the cryostat set at -24°C for incubation for one hour. The tissue was then cut serially at 50µm. The slides were left at RT o/n to dry before being labelled.

Tissue collection: adult animals

Adult animals were collected at three months of age. Upon collection, animals were injected with sodium pentobarbital (0.1ml per animal) and decapitated. Their tongues were then dissected, washed once in PBS and put to 4% PFA o/n at 4°C. Samples were washed in PBS and placed in 30% sucrose at 4°C o/n. After this step the procedure was identical to above-described younger animals. The tissue was then embedded in OCT (Mounting medium for cryotomy, VWR). First, plastic moulds (Peel-A-Way Disposable Embedding Molds, Polysciences, USA)

were filled with liquid OCT, which was then put on dry ice. When the bottom layer of OCT was frozen solid, the tissue was placed in the middle of the mould, which was then immersed in dry ice so that the moulds would be covered on all sides except for the open top. After a few hours on dry ice the samples were transferred to -80°C.

When the tissue needed to be cut, the samples were transferred from the -80°C to the cryostat set at -24°C for incubation for one hour. The tissue was then cut serially at 50µm. The slides were left at RT o/n to dry before being labelled.

Immunohistochemistry

E16.5 and P0 animals

After drying, half of the sections were taken for staining, so that the tongue would be represented by a section every 100µm, while the reset was taken to storage at -80°C.

The sections were first washed 4 x 15 minutes in 0.1M PB. They were then blocked o/n at 4°C in blocking solution (3% NGS, 0.5% Triton-X 100, 0.1% NaN_3 in .1M PB). The next day the sections were incubated in primary antibody solution (200 µl per slide): TUJ1 (1:300) and P2X3 (1:500) (for E16.5 samples); or Troma-I (1:200), TUJ1 (1:300) (in P0 samples); 0.5% Triton-X 100, 0.1% NaN_3 in 0.1M PB. The slides were then covered with parafilm (Pechiney, USA) to prevent drying, and left at 4°C for 5 days. After the primary antibody incubation, the slides were washed again 4 x 15 minutes in 0.1M PB and put into secondary antibody solution (Alexa-488 anti-mouse (1:1000) and Alexa-555 anti-rabbit (1:1000) (for E16.5 samples); or Jackson FITC anti-rat (1:1000), Alexa-555 anti-mouse (1:1000) (for P0 samples); 0.5% TritonX-100, 0.1% NaN_3 in 0.1M PB) o/n at 4°. The next day the slides were washed with 0.1M PB, 4 x 15 mins, and mounted in Vectashield without letting the tongues dry.

The stained tongues were then imaged under a Zeiss LSM710 Meta confocal microscope with a plan-apochromat 20x/0.8 M27: a z-stack of images every 4µm

was taken of a tile scan of 3x7 tiles. This imaged the whole section of the tongue, so that the innervation into the neural/taste buds was possible to be analyzed.

Adult animals

After drying, half of the sections were taken for staining, so that the tongue would be represented by a section every 100µm, while the reset was taken to storage at -80°C.

The sections were then washed 4x15 minutes in 0.1M PB, followed by an antigen retrieval procedure. In this case, each slide was subjected to 10 minutes of 200µl of proteinase K in 0.1M PB at 20µg/ml. This was followed by a wash in 0.1M PB 2x 10 minutes and quenched in Dario's solution (0.05% NaN_3, 0.1M glycine, 0.1M NH_4Cl, 50mM Tris) for 30 minutes at RT. The slides were then washed 2x15 minutes in 0.1M PB. After this step the procedure was the same as the one used for P0 samples. The slides were blocked o/n at 4°C in blocking solution (3% NGS, 0.5% Triton-X 100, 0.1% NaN_3 in .1M PB) and the next day thes were incubated in primary antibody solution ((200 µl per slide): Troma-I (1:200), TUJ1 (1:300), 0.5% Triton-X 100, 0.1% NaN_3 in 0.1M PB). The slides were then covered with parafilm (Pechiney, USA) to prevent drying, and left at 4°C for 5 days. After the primary antibody incubation, the slides were washed again 4 x 15 minutes in 0.1M PB and put into secondary antibody solution (200µl per slide of Jackson FITC anti-rat (1:1000), Alexa-555 anti-mouse (1:1000), 0.5% TritonX-100, 0.1% NaN_3 in 0.1M PB) o/n at 4°. The next day the slides were washed with 0.1M PB, 4 x 15 mins, and mounted in Vectashield without letting the tongues dry.

The stained tongues were then imaged under a Zeiss LSM710 Meta confocal microscope with a plan-apochromat 10x/0.45 M27: a z-stack of images every 8µm was taken of a tile scan of 4x6 tiles. This imaged the whole section of the tongue, so that the innervation into the neural/taste buds was possible to be analyzed.

Analysis of tongue innervation

Using ImageJ the tongues were then scanned for the presence of innervated taste buds. ImageJ enables first splitting and subsequently merging the channels of the image, which was used to look at overlay of the tongues. In this way we were able to assess the presence of any taste buds, either innervated or uninnervated (in the case of P0 pups), on each section. Each section was also split into a "tip" and a "middle" using ImageJ "Straight" function (Figure 6), so that the anterior 2/3 of each tongue were halved still. All the taste buds were then counted; if the whole taste bud was not encompassed within a single section we counted this as one half. The count for the whole tongue was then multiplied by two, which gave the total count of taste bud in the whole tongue, tip, and the middle.

Analysis of taste bud morphology

Tongue sections previously use for innervation analysis were examined for taste buds morphology. Taste buds were selected at random, one from each section if possible, with their placement on the tongue noted in order to establish the analysis of the tip and middle parts of the tongue. High magnification images were taken with Zeiss LSM710 Meta confocal microscope using the plan-apochromat 63x/1.40 Oil DIC M27 objective. Images were taken every 1µm scanning for both 555nm and 488nm wavelengths. Images were then analysed with ImageJ. Using the function "Z Project" the images were amalgamated into maximum intensity, and the widest and highest points were measured using the "straight" tool. These were then recorded as width and height of the taste buds. Identical analysis was performed on P0 taste buds as well as adult taste buds.

DiI labelling

DiI labelling was performed on embryos aged E14.5. The litter was first placed on ice for ten minutes. The embryos were perfused with 4% paraformaldehyde under the microscope and placed in 4% PFA at 4°C o/n. The next day the embryo was labelled with DiI (Invitrogen): the entire embryo was left intact so that it can be moved without having its head touched.

Because of the size of the embryo, the procedure is done under a Leica Stereomicroscope (MZ125). The embryo is pinned down, and a pair of forceps is placed under the chin to hold its head up. Next, the top of the head is cut off with a pair of small scissors. It is critical that the cut is not made too far down the head otherwise the geniculate ganglia will be damaged. The brain is removed, so that the only nervous tissue visible in the skull is the trigeminal ganglion. The trigeminal ganglion is removed because it is important for geniculate ganglion to be the only one projecting into the anterior tongue. The most caudal portion of the trigeminal ganglion rests on a boney ridge: the procedure needs to be carried out carefully in order not to touch this ridge because it contains the geniculate ganglion. Removing the trigeminal ganglia exposes the facial nerve entering the skull through foramen in the back of the boney ridge. DiI crystals are placed on this nerve in a quite a large volume, and any stray particles are cleaned up with the forceps. The animal is placed in a water-soaked towel for half an hour before it is placed back into the 4% PFA. The embryos are then incubated at 37°C for 4 weeks, with one PFA exchange after one week.

When the DiI transport is finished (the animals are periodically checked under a fluorescent dissectoscope every week), the tongue is dissected out carefully in order to include the whole tongue. It is then placed on a microscope slide, and attached to the slide by putting a drop of low-melt agarose onto the tongue. Once the agarose gel is set, the tongue is scanned under the confocal microscope using laser at 488 wavelength, at 10x. This forms a Z-stack of the anterior tongue (we only included the part of the tongue anterior to the circumvallate papilla), which can then be examined using Imaris.

DiI labelling analysis

Using Imaris function "filament", the innervation in the tongue was manually traced and then evaluated in terms of branch thickness, total innervation, number of branches and branch length, which could then be compared across genotypes, a method that has been done before (Ma et al. 2009). In each tongue a stereotypical area in the anterior medial part of the tongue would be designated for analysis. Unfortunately, using Imaris showed wild inaccuracy of the DiI method. Tongues labelled with this dye very rarely showed labelling into the whole tongue: often only certain regions of the tongue were labelled only, such as only the tip of the tongue showing labelling of the dye but not the more anterior part. Several times we also observed only one half of the tongue being labelled with the other half of the tongue being blank. Most of our tongues were also plagued by a large amount of background stain, caused most likely by the dye shifting from the place of label to different regions of the embryonic head during incubation. Presumably, this was caused by the inconsistency of labelling of the facial nerve by the dye. We therefore abandoned this method as one used for determining the amount of innervation into the tongue.

Whole mount labelling of mouse embryo

Embryos aged E16.5 were obtained and incubated on ice for 10 minutes. The tongues were dissected and immersed in Dent's fixative (20% DMSO, 80% methanol) o/n at 4°C in NUNC tubes, followed by bleaching o/n in 1:2 in H_2O_2: Dent's fixative at RT. The tongues were washed 3x20 mins in TBS, and incubated in blocking solution (4% calf serum (Sigma) in DMSO, 3µM Thimerosal (Sigma)) and TUJ1 (1:300) o/n at RT. The tongues were flushed several times with TBS, then washed in TBS 5x 1hr each at RT. Incubation in blocking solution with anti-mouse HRP-conjugated antibody (1:300) (Abcam) o/n at RT on a shaker (Stuart Gyro-rocker SSL3). The tongues were then flushed several times with TBS and washed 5x1hr each with TBS at RT. They were then reacted with DAB working solution (1 tablet of

each dissolved in 1ml dH_2O (Sigma) for 20 minutes, until the tongues looked brown. Samples were washed several times with TBS. Then they were incubated for 30 minutes in TBS: methanol 1:1, 2x 30 minutes in 100% methanol, methanol: BABB 1:1 for 5 minutes, 100% BABB. BABB made the tissue transparent. When taking pictures the tongue was put onto a microscope slide with some BABB, a low-melt agarose (melted) was dropped onto the tongue and pictures were taken immediately using a brightfield microscope (Zeiss Axio Scope.A1) using a 63x/1.4 lens and a Zeiss AxioCam 1Cc1 camera.

After imaging more BABB was put on top of the sample for melting of the agarose. The previous procedure was then done backwards (100% BABB, BABB: methanol 1:1, 100% methanol, TBS : methanol, TBS), and the samples were stored at 4° in TBS.

DAB stain

DAB stain was performed on tongue OCT sections up to 25µm thick. After sectioning, the slides are left to dry o/n at RT. They are then washed in PB 3x15 minutes to wash OCT away, followed by bleaching in 2% H2O2 in dH2O. The slides are washed 2x5 minutes in TBS and blocked (10% NGS, 1% BSA, 0.5% Triton-x100, TBS) for 1hr at RT. The samples are incubated in primary antibody solution (1% NGS, 1% BSA, 0.1% Triton-X100, TBS) o/n at 4°C. After washing 3x5 minutes in TBS, the tongues are incubated in secondary antibody solution 1:200 (biotinylated secondary antibodies, Vector labs) in (2.5% BSA, 0.5% Triton-X100, TBS) for 2 hours at RT, followed by a wash 3x5 minutes in TBS. Samples are then incubated for 30 minutes in peroxidase Vectastain ABC system from Vector Labs (1:100 of reagent A and B in TBS, prepared 30 minutes in advance in order to form avidin-biotin-peroxidase complexes, since the system contains avidin and biotinylated peroxidise). This incubation causes the free biotin-binding sites of avidin to bind to the biotinylated secondary antibody on the sections. The sections were washed in TBS 3x5 minutes and TB (50mM, pH8.3) 2x5 minutes. For developing we used the DAB (3'3-diamobenzidine) from Sigma, where tablets dissolved in water create a ready-to-

use solution. In this reaction, DAB is converted to a brown precipitate by the peroxidise in the presence of hydrogen peroxide. Sections were incubated from 20 seconds to 5 minutes depending on the speed of the sections turning brown. To stop the colour reaction, 2x5 minute wash in ice-cold TB was used, followed by 90% EtOH and 100% EtOH for 5 minutes each. Slides were then incubated in xylene for 10 minutes before being mounted using DPX and dried o/n under the fume hood. Images were taken immediately using a brightfield microscope (Zeiss Axio Scope.A1) using a 63x/1.4 lens and a Zeiss AxioCam 1Cc1 camera.

2.6 Statistical analysis

All experiments were carried out blind to the genotype. The results are indicated as mean ± standard deviation. Significance between samples was calculated using Student's t-test, one-way and two-way ANOVA and Fisher's PLSD post-hoc test. Differences were considered significant when $p<0.05$.

3. RESULTS

3.1 Geniculate ganglion neuron survival

3.1.1 TrkB/Shc docking site point mutation causes loss of geniculate ganglion neurons at E14.5; point mutation in the TrkB/PLCγ docking site does not

BDNF and NT4 have been shown to exert different influences on geniculate ganglion neurons throughout rodent gustatory development, although these influences fluctuate with developmental stages as does the expression of these neurotrophins in gustatory system. The initial phase of this study set out to determine which of the two aforementioned adaptor sites, TrkB/Shc and TrkB/PLCγ1, were able to facilitate the influence of either BDNF or NT4 on the neurons in the geniculate ganglia. The primary point of influence of both neurotrophins on the geniculate ganglion neuron survival was shown to be at embryonic day 14.5 (E14.5), when null mutations in either *Bdnf* or *Nt4* were shown to cause loss of about a half of all neurons in the geniculate ganglion (see Introduction Chapter 2, Conover et al. 1995, Liu et al. 1995, Patel and Krimm 2010a). Because *Trkb* null mice have been shown to lose nearly all of the geniculate ganglion neurons by this stage (Fritzsch et al. 1997), it has been accepted that survival of these neurons is facilitated primarily by this receptor. In order to determine which adaptor site on TrkB receptor facilitates the regulation of the geniculate ganglion neurons, we examined the survival of geniculate ganglion neurons at E14.5 in all of the mutants.

At this stage of development, mutation in the TrkB/Shc adaptor site had a profound influence on the geniculate ganglion neurons. By counting the neuronal cell bodies (see Methods) we found that the $Trkb^{S/S}$ embryos had significantly lower number of geniculate ganglion neurons compared to their wild type counterparts: 398 ± 142 vs. 915±81, respectively, both n=3, $p < 0.001$ (E14.5, Figure 5A, H, I).

On the other hand, the TrkB/PLCγ adaptor site did not show such an effect. The $Trkb^{P/P}$ embryos showed similar number of geniculate neurons compared to the

wild type control embryos (878±77 vs. 815.5±67, respectively, p=0.279, *Trkb*$^{P/P}$ n=6; *Trkb*$^{W/W}$ n=3), (E14.5, Figure 5B, J, K). Mutation at both adaptor sites had a profound effect on the neuronal survival in the geniculate ganglia as *Trkb*$^{D/D}$ embryos, similarly to what has been found in Trkb null embryos at this stage (Fritzsch et al. 1997), lost on average 90% of their neurons; geniculate ganglion in these mutants were significantly smaller than the wild type controls with only 84±5 neurons per ganglia compared to 815.5±67, p <0.001, n=3 (E14.5, Figure 5B, L).

These data indicate that the TrkB/Shc docking site is involved in the regulation of geniculate ganglion neuron survival, and that it is the main site transducing BDNF and NT4 survival signalling by this age (E14.5). The point mutation in the TrkB/PLCγ docking site, on the other hand, was found not to cause any loss of geniculate ganglion neurons. Because of the large amount of geniculate ganglion neuronal loss in double mutant *Trkb*$^{D/D}$ animals, the TrkB/PLCγ docking site may play a supporting role in geniculate ganglion survival rather than have a direct influence on neuronal loss. Furthermore, the loss of neurons in *Trkb*$^{D/D}$ animals highlighted the importance of signalling pathways downstream of TrkB for regulation of geniculate ganglion neuron survival.

3.1.2 TrkB/Shc docking site point mutation causes loss of geniculate ganglion neurons at E12.5; point mutation in the TrkB/PLCγ docking site does not

Neurotrophin influence on geniculate ganglion neuron survival starts early in gustatory development. NT4 initially determines the number of surviving neurons by E12.5, while BDNF does not exert its influence until E14.5. In order to separate the survival effect of neurotrophins on the geniculate ganglion neurons and to determine whether the TrkB/Shc docking site is able to facilitate the influence of NT4 early in gustatory development, the geniculate ganglion neuronal populations in all genotypes were examined at embryonic day E12.5

Neuronal counts at this stage revealed that the *Trkb*$^{S/S}$ animals had significantly lower number of neurons than their wild type counterparts: 702±47 vs. 869±51, p <0.01, *Trkb*$^{S/S}$ n=3, *+/+* n=4 (E12.5, Figure 5A, C, D).

On the other hand, similar to the data obtained at E14.5 stage, mutation at the TrkB/PLCγ adaptor site did not influence the survival of geniculate ganglion neurons at E12.5; the *Trkb*$^{P/P}$ animals' geniculate ganglion neuron number did not differ from the wild type control animals (935±7 vs. 937±74, respectively, p=0.975, *Trkb*$^{P/P}$ n=2, *Trkb*$^{W/W}$ n=4, E12.5, Figure 5B, E, F), although in this case, due to time contraints, we were only able to examine two *TrkB*$^{P/P}$ animals.

As expected, the double adaptor site mutation, *Trkb*$^{D/D}$, caused larger loss than we found in any of the other examined genotypes (183±27 *TrkB*$^{D/D}$ n=3 compared to 935±7 (*Trkb*$^{P/P}$ n=2) and 937±74 (*Trkb*$^{W/W}$ n=4)); significantly lower than the wild type control (p <0.001) and the *Trkb*$^{P/P}$ animals (p <0.001) (E12.5, Figure 5B, G). The loss, however, wasn't as extensive as it was seen at later developmental stage: 90% loss at E14.5, and 80% at E12.5 compared to the wild type control geniculate ganglion neurons.

These results suggest that it is therefore largely the TrkB/Shc docking site that facilitates neuronal loss in the geniculate ganglion at this stage, while the TrkB/PLCγ docking site appears to play only a supportive role in this process. This is due to no loss of geniculate ganglion neuron loss seen in *Trkb*$^{P/P}$ animals but a large amount of loss in *Trkb*$^{D/D}$ animals which is more extensive than that seen in *Trkb*$^{S/S}$ animals. This also suggests a crucial role of TrkB signalling in early stages of geniculate ganglion development. Because only NT4 is able to cause loss of these neurons at this stage, we have concluded that the TrkB/Shc docking site facilitates signalling of NT4 that influences the geniculate ganglion neuron survival early in gustatory development.

3.1.3 Point mutation at the TrkB/Shc adaptor site influences the loss of geniculate ganglion neurons after embryonic development, no losses are seen in animals with TrkB/PLCγ mutation

Having established that the TrkB/Shc site is the adaptor site facilitating the survival signalling of geniculate ganglion neurons during the embryonic development of the gustatory system, we wanted to examine the neuronal survival of these neurons at the end of the development of the gustatory system, to see whether the deficits in neuronal survival persist after the embryonic development is completed. We therefore examined the neuronal survival of the geniculate ganglion at postnatal day 4 (P4). We found that the mutation at the TrkB/Shc site caused a further loss of neurons in the geniculate ganglion compared to their wild type counterparts (261±32 vs. 789±38, respectively, $Trkb^{S/S}$ n=2, *+/+* n=4, $p < 0.001$), (P4, Figure 5A M, N). This reduction was seen despite the fact that we were unable to obtain sample size large enough in time to complete this experiment.

Animals carrying a point mutation in the TrkB/PLCγ site, similarly to what has been found at embryonic stages, did not show significantly different amount of surviving geniculate ganglion neurons when compared to geniculate ganglia of wild type control animals: 790±76 vs. 799±37, respectively (both n=3, p=0.852), (P4, Figure 5B, O, P).

Taken together, data from these experiments show that the TrkB/Shc adaptor site, and thus the signalling pathways activated through this site downstream of the TrkB receptor, is involved in regulating survival of the geniculate ganglion neurons as facilitated by both BDNF and NT4. Despite the fact that $Trkb^{P/P}$ animals showed no loss of geniculate ganglion neurons, we would not expect the TrkB/PLCγ adaptor site to be dispensable to this process, but rather to play a supportive role in survival of at least a subpopulation of neurons, because the losses of geniculate ganglion neurons seen in *Trkb*-null mice in previous experiments (Conover et al. 1995, Fritzsch et al. 1997).

Double mutants were not examined at this stage of development because *Trkb*$^{D/D}$ animals are not viable and die shortly after birth. Due to no recovery of the geniculate ganglion neurons seen in the *Trkb*$^{S/S}$ animals and to previous findings showing loss of geniculate ganglion neurons in newly born (and just before birth) *Trkb* null and *Bdnf/Nt4* null animals (Conover et al. 1995, Liu et al. 1995, Fritzch et al. 1997), we would expect no recovery in the *Trkb*$^{D/D}$ animals to be seen at the end of the embryonic development around birth.

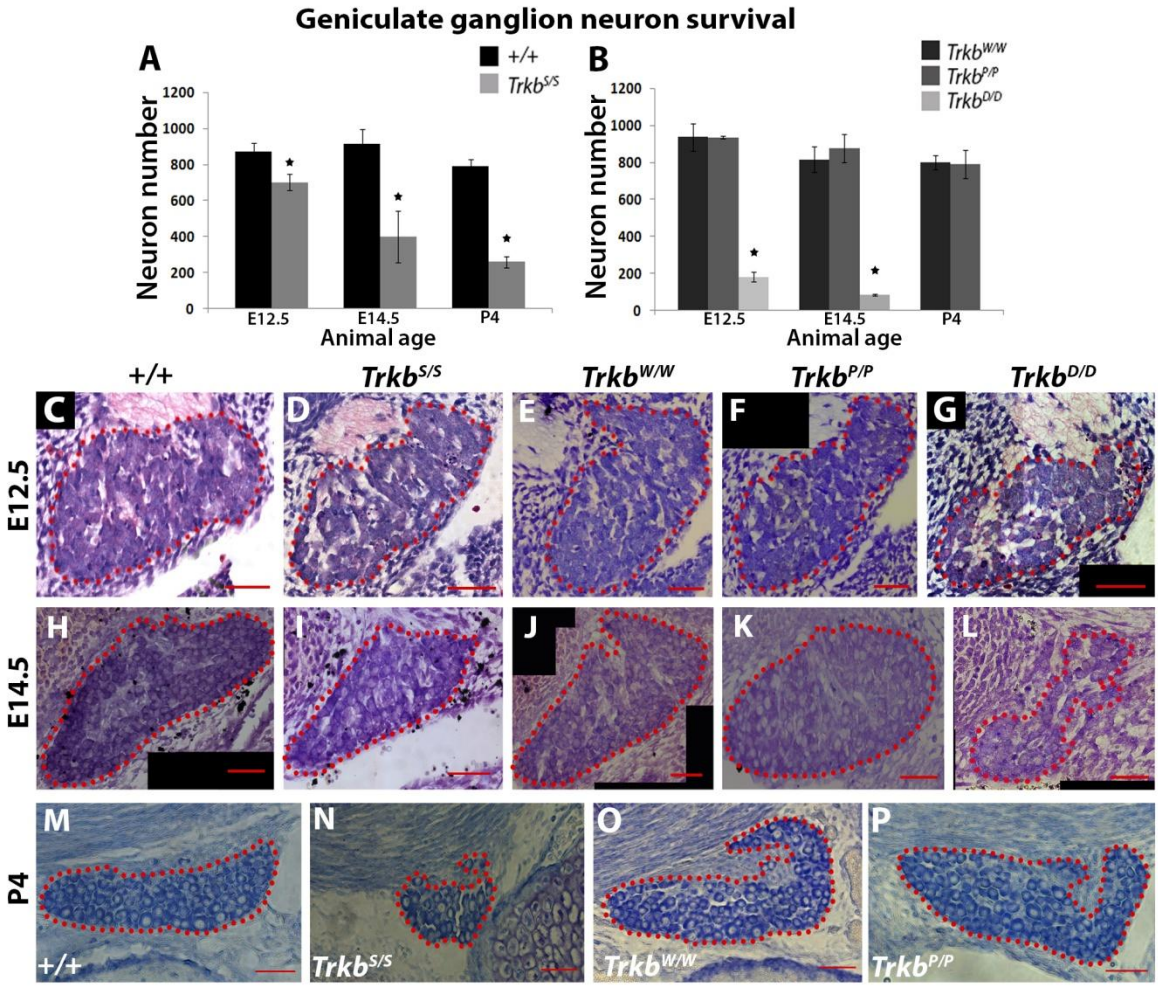

Figure 5: Comparison of geniculate ganglion neuron survival between different genotypes throughout the gustatory development. **A**, *Trkb*$^{S/S}$ animals show reduced number of surviving neurons compared to control animals at all stages analysed. **B**, *Trkb*$^{P/P}$ animals show no difference compared to wild type control animals, *Trkb*$^{D/D}$ animals have reduced number of geniculate ganglion neurons at all stages analysed. **C-G**, Representative images of Haematoxylin-Eosin-stained sections of the geniculate ganglion of all genotypes at E12.5. **H-L**, Representative H-E-stained sections of the geniculate ganglion of all genotypes at E14.5. **M-P** Cresyl Violet-stained sections of geniculate ganglion of *+/+*, *Trkb*$^{S/S}$, *Trkb*$^{W/W}$ and *Trkb*$^{P/P}$ at P4. All scale bars are 50µm. Significant differences were calculated using Student's t-test (**A**), and two-way ANOVA (**B**).

3.2.1 Point mutation at the TrkB/PLCγ adaptor site decreases the amount of innervated neural buds on the tongue during embryonic development

The innervation pathways from the geniculate ganglion into the tongue are influenced by the actions of neurotrophins. At E16.5, two days after the initial innervation into the tongue, the *Bdnf* null mutation causes loss of innervation of neural buds. *Nt4* null mutation, on the other hand, does not appear to have a role at this age (Krimm et al. 2001). In order to understand whether the TrkB/PLCγ adaptor site, which had no effect on survival, would instead facilitate the process of innervation mediated by BDNF we examined the innervation into the tongue in $Trk^{P/P}$, $Trkb^{W/W}$ and $Trkb^{D/D}$ mouse embryos at E16.5. $Trkb^{S/S}$ mice were not examined at this stage as we would expect low amount of surviving geniculate ganglion neurons to cause low amount of innervating fibers into the tongue.

As mentioned previously, the geniculate ganglion afferents innervate the fungiform papillae that are present on the anterior-most two thirds of the tongue. We decided to examine the total amount of innervated neural buds as well as the possibility of a difference between the different regions on the tongue. The tongue was therefore split into two different regions, the tip and the middle, which corresponded to the posterior-most end of the "middle" region ending proximally to the circumvallate papilla (Figure 6, shows the divisions of the tongue at different stages: A, E16.5; B, P0; C, adult mouse tongue). At this age, in order to standardise the measurements, the tip of the tongue was defined as the anterior-most 1250µm of the tongue, while the middle part of the tongue as an area following the tip of the tongue from 1250-2500µm (Figure 6A, tongue divisions at E16.5).

Immunohistochemical analysis made use of antibodies used in experiments previously; anti-ß-III tubulin antibody,Tuj1, showing innervation into the taste buds and anti-P2X3-receptor antibody for ATP receptors that are located on the gustatory afferents on the tongue (Al-Hadlaq et al. 2003, Qian et al. 2006, Ishida et al. 2009, Nosrat et al. 2012).

The analysis of the tongue sections examining the total innervated neural buds showed that *Trkb*$^{P/P}$ embryos contained significantly lower amount of innervated neural buds on their tongues than the wild type control animals (54±17.4 vs. 113.3±8.3, n=3 for both genotypes, p <0.01), while the innervation in *Trkb*$^{D/D}$ animals (33.3±5, n=3) was lower than the wild type control animals (p <0.001), but not compared to *Trkb*$^{P/P}$ animals (p=0.119) (Figure 7A, E-M).

However, by looking at the different regions of the tongue we found that while the amount of innervated neural buds in the anterior part of the tongue (tip) showed significant differences between controls and mutants (*Trkb*$^{W/W}$=85.3±5.8, n=3, and *Trkb*$^{P/P}$=35.3±18.6, n=3, p <0.01 and *Trkb*$^{W/W}$ and *Trkb*$^{D/D}$=31.3±4.2, n=3, p <0.001), the mutants (*Trkb*$^{P/P}$ and *Trkb*$^{D/D}$) were not significantly different from each other(p=0.734, Figure 8B, D, F, H). This was different in the middle part of the tongue. Although the amount of innervated neural buds in *Trkb*$^{D/D}$ animals was still significantly lower than in the wild type controls (2±2 vs. 28±6, p <0.001), *Trkb*$^{P/P}$ animals' innervated neural bud counts were not significantly different from the wild type controls (*Trkb*$^{P/P}$=18.7±3.1, *Trkb*$^{W/W}$= 28±6, p=0.074, Figure 8C, E, G, I). This suggests that the TrkB/PLCγ adaptor site only had an influence on the innervation at this developmental stage in the anterior-most part (tip) of the tongue, but not in the middle, even though the fungiform papillae in both areas are innervated by the geniculate ganglion. This could mean that the neural buds present in the tip of the tongue are more susceptible to loss of neurotrophin survival, although the actual mechanism of this process will need to be clarified further.

Overall, these results suggest that because we did not find any deficits in the amount of surviving geniculate ganglion neurons in the Trkb$^{P/P}$ animals, deficits in innervation in these animals likely reflects alteration in guidance of the geniculate ganglion fibers into their target areas rather than an increased amount of axons from the geniculate ganglion into the tongue. The double mutation, showing the largest deficits in both innervation and geniculate ganglion survival would therefore likely show deficits due to the lack of sufficient amount of fibers to enter the tongue, together with deficient targeting.

Next, we wanted to determine whether the point mutations in the adaptor sites alter the amount of taste buds present in different designated areas: the tip and the middle. To do this we counted the amount of innervated neural buds in each area and expressed them as a proportion of the total amount of innervated neural buds.

While the tip area contains 75% of all innervated neural buds in the wild type control animals, this area contains 63% of all innervated neural buds in *Trkb*$^{P/P}$ animals (Figure 8C). The difference in this case is not statistically significant (p=0.215) between the two genotypes as both show higher proportion of innervated neural buds present in the tip of the tongue. On the other hand, in *Trkb*$^{D/D}$ animals, this area represents 94% of the neural buds (Figure 8C). The amount of innervated taste buds in the tip of the tongue the double mutant animals is significantly higher compared to the wild type controls (p=0.011) as well as to the *Trkb*$^{P/P}$ animals (p=0.023), suggesting that TrkB signalling affects not only the amount of innervated taste buds, but also the localization of these taste buds on the tongue.

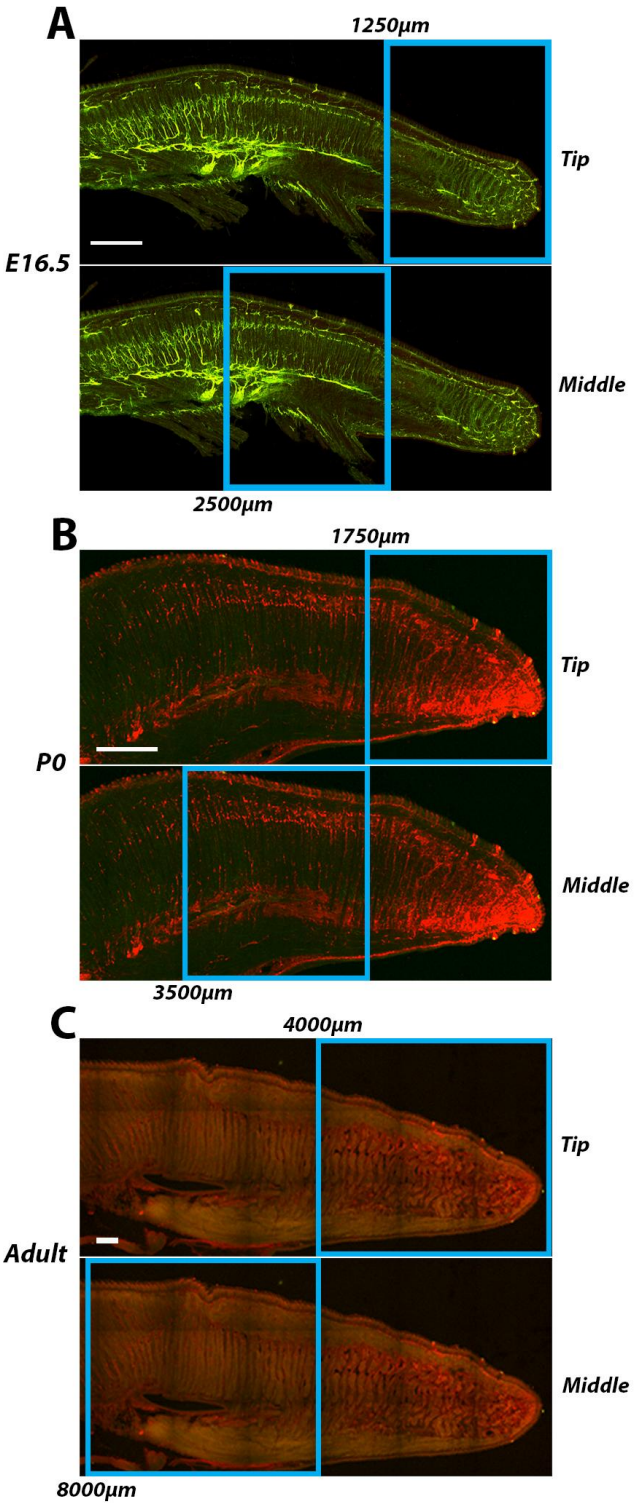

Figure 6: Division of tongue into tip and middle for innervation analysis in embryonic mice at E16.5, **A** (merged stains of Tuj1 (green) and P2X3 (red)), newly born animals **B**, and adult animals, **C** (merged stain of Tuj1 (red) and Troma-I (green)). Blue boxes represent areas that were considered tip and middle parts of the tongue for each stage. All scale bars are 400µm.

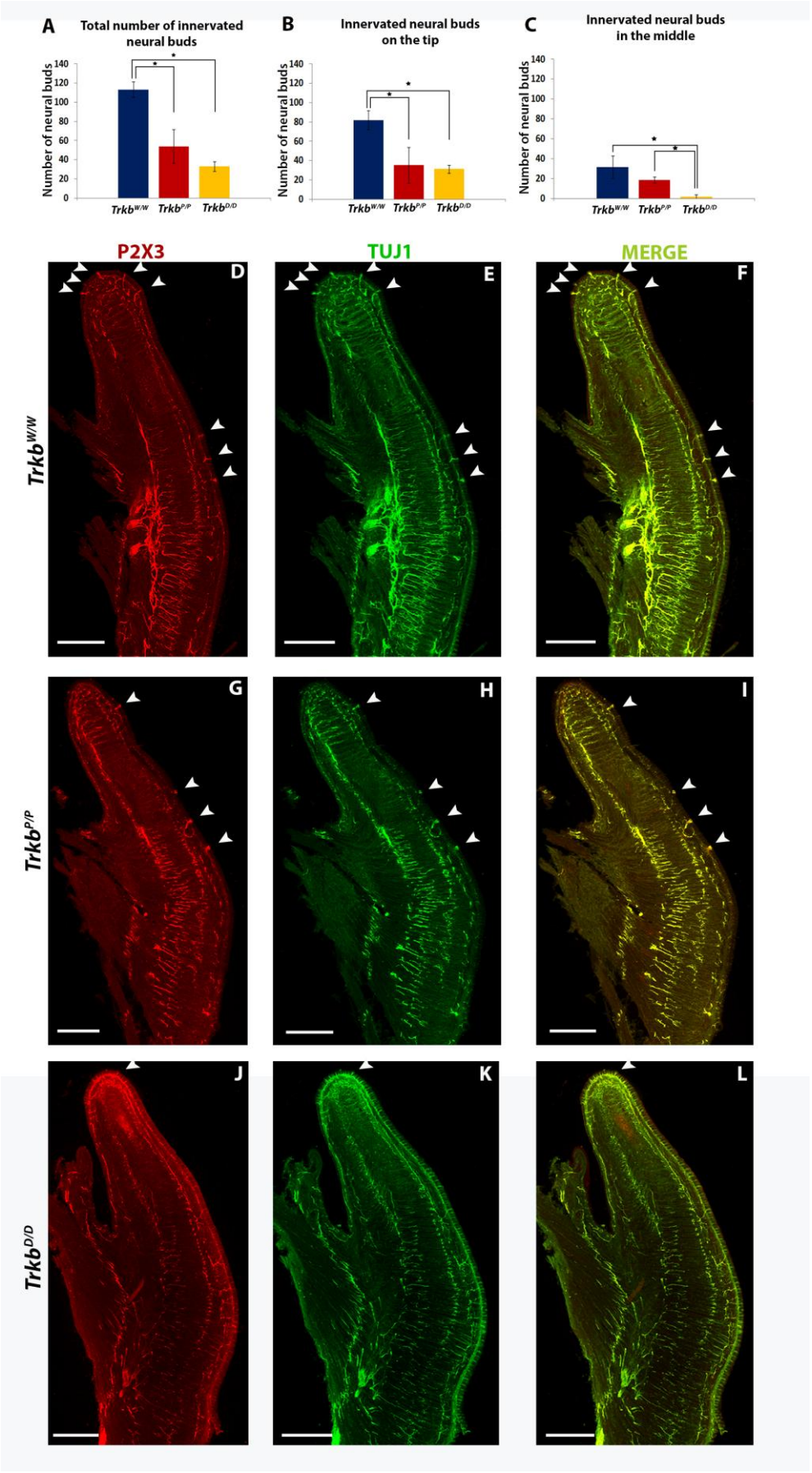
A
Total number of innervated neural buds
Number of neural buds
140
120
100
80
60
40
20
0
TrkbW/W
TrkbP/P
TrkbD/D
B
Innervated neural buds on the tip
Number of neural buds
C
Innervated neural buds in the middle
Number of neural buds
P2X3
TUJ1
MERGE
D
E
F
G
H
I
J
K
L
TrkbW/W
TrkbP/P
TrkbD/D

Figure 7 (on previous page): Analysis of tongue innervation at E16.5. *Trkb*$^{P/P}$ and *Trkb*$^{D/D}$ embryos contained lower amount of innervated neural buds on whole tongues compared to wild type control embryos, **A**. **B-J** show representative sections of tongues of all animals stained with P2X3 (**B, E, H**), Tuj1 (**C, F, I**) and merge images of both (**D, G, J**). Arrowheads indicate presence of innervated neural buds. Scale bars 400µm. Statistical difference was calculated using a two-way ANOVA.

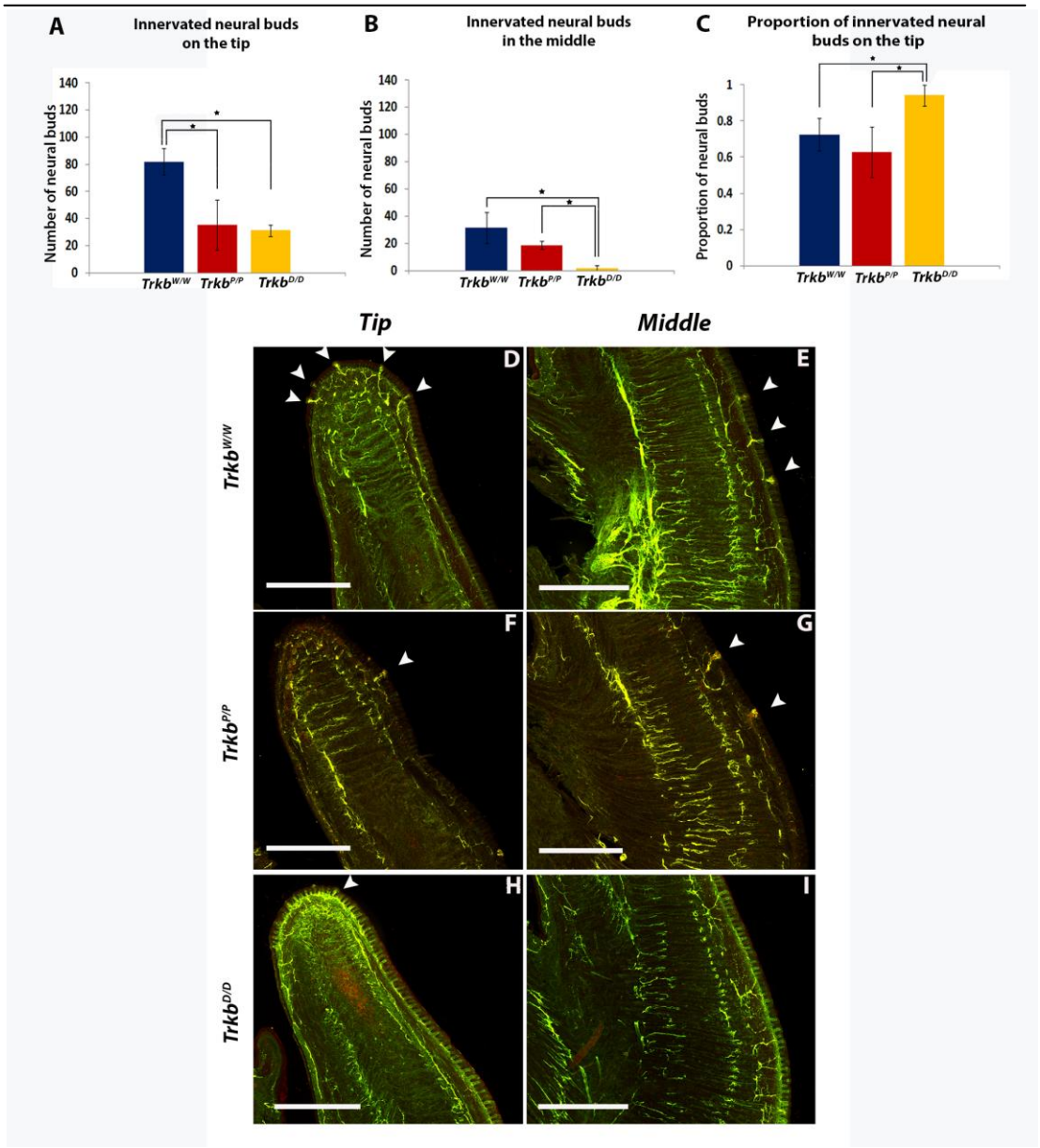

Figure 8: Analysis of tongue partitions at E16.5**.** Both mutant animals contained lower amount of innervated neural buds in the tip of the tongue, **A**. *Trkb*$^{P/P}$ embryos showed no difference in the middle of the tongue compared to *Trkb*$^{W/W}$ animals, while the *Trkb*$^{D/D}$ animals had lower numbers of innervated neural buds, **B**. Proportion of innervated neural buds on the tip of the tongue was higher in *Trkb*$^{D/D}$ animals compared to both *Trkb*$^{W/W}$ and *Trkb*$^{P/P}$ animals at E16.5, **C**. **D, F**, **G** show high magnification of tip of the tongue of and **E**, **G**, **I** high magnification of the middle of the tongue of *Trkb*$^{W/W}$, *Trkb*$^{P/P}$ and *Trkb*$^{D/D}$ animals, respectively. All images represent merged staining of Tuj1 and P2X3. Arrowheads indicate presence of neural buds. All scale bars are 400µm. Significant difference between proportions of taste buds was calculated using two-way ANOVA.

3.2.2 *Trkb*$^{P/P}$ mice show increased innervation in the tongue after embryonic development, while the *Trkb*$^{S/S}$ mice show a deficit

Neural buds are considered to have developed into functional taste buds after birth. Therefore, examination of innervation once the taste system is developed but not yet completely matured, such as in newborn animals at postnatal day 0 (P0), would show possible innervation differences at the end of development. Maturation of the taste buds in the mouse occurs after birth and persists for a long period of time and their innervation may vary from case to case. In order to determine whether the Trkb/Shc and TrkB/PLCγ docking sites influence the innervation into the tongue also after the birth of the animal we examined the amount of innervated as well as uninnervated taste buds (uninnervated taste buds are discussed in Section 3.2.3) that are present on either the whole tongue or the two previously defined areas of the tongue.

At P0 we were able to use a different immunohistochemical approach to that used in animals at a younger age. While the innervation into individual taste buds was still visualised using the anti-ß-III tubulin antibody (Tuj1), taste bud-specific antibody assaying for cytokerain 8, Troma-I, was used to specifically identify the presence of taste buds (Ito et al. 2009, Nosrat et al. 2012). As with the younger animals, we evaluated the amount of innervated taste buds present on the whole tongue (area representing up to 3500µm from the tip of the tongue), or only the tip part of the tongue (up to 1750µm from the tip of the tongue), and middle part of the tongue (following the tip from 1750-3500µm) at P0 (Figure 6B) for all the genotypes where the posterior-most border of the "middle" area touched the circumvallate papilla present on the tongues of these animals.

The *Trkb*$^{P/P}$ animals showed an increase in innervated taste buds present on their tongues when compared to the wild type control animals (163.2±7.8 vs. 122.2±17.1, respectively, *Trkb*$^{P/P}$ n=5, *Trkb*$^{W/W}$ n=6, $p < 0.001$). *Trkb*$^{D/D}$ animals, on the other hand, showed a much lower amount of innervated taste buds on their

tongues (38.7±7.57 n=3) compared to both the wild type control animals ($p < 0.001$) and the *Trkb*$^{P/P}$ animals ($p < 0.001$, Figure 9A, B-J).

Higher amount of innervation in the *Trkb*$^{P/P}$ animals was also found when we compared the number of innervated taste buds in the tip of the tongue, where *Trkb*$^{P/P}$ animals showed higher amount of innervation (133.6±9.2) compared to the wild type controls (101.3±18.2), $p < 0.01$. The innervation in the tip of the tongue of *Trkb*$^{D/D}$ animals (29.3±7.6) was, however, still much lower than both the control animals ($p < 0.001$) and the *Trkb*$^{P/P}$ animals ($p < 0.001$, Figure 10A, D, F, H).

Very similar effect was seen in the middle parts of the tongue. The amount of *Trkb*$^{P/P}$ animals' innervated taste buds present in middle areas of the tongues was significantly higher than that in wild type control animals (29.9±3.6 vs. 20.8±4.8, respectively, $p < 0.01$). The *Trkb*$^{D/D}$ animals' middle areas were, however, still lower in amount of innervated taste buds (9.3±7.6) than the *Trkb*$^{P/P}$ animals ($p < 0.01$) as well as the wild type control animals ($p=0.025$) (Figure 10B, E, G, I). These results suggest that the TrkB/PLCγ adaptor site has a profound influence on the taste bud innervation at P0, as we have seen a large increase in innervated taste buds in the whole tongue, as well as in the two previously defined areas compared to both the wild type control animals, and the innervation seen in *Trkb*$^{P/P}$ embryos at an earlier stage (*Trkb*$^{P/P}$: 54±17.4 at E16.5 and 163.2±7.8 at P0, $p<0.001$). Interestingly, the amount of innervation into the neural/taste buds did not change in wild type control animals or in *Trkb*$^{D/D}$ animals between E16.5 and P0 (*Trkb*$^{W/W}$: 113.3±8.3 at E16.5 and 122.2±17.1 at P0, $p=0.435$; *Trkb*$^{D/D}$: 33.3±5 at E16.5 and 38.7±7.57 at P0, $p=0.367$), suggesting that the mutation in the TrkB/PLCγ adaptor site is able to recover the amount of lost innervation at an earlier age and even exceed the wild type control animals in this aspect while the amount of innervation in the control animals remains constant. The innervation in double mutant *Trkb*$^{D/D}$ animals was very low, likely reflecting the low amount of surviving geniculate ganglion neurons found in earlier developmental stages.

As expected from the survival analysis of the geniculate neurons, *Trkb*$^{S/S}$ animals' innervated taste buds were significantly lower in number than those

present in wild type littermates in the whole tongue (56.3±3.5 vs. 142±11.5, respectively, n=3 in both cases, p <0.001, Figure 11A, B-J), tip area of the tongue (52.3±3.2 vs. 117±10.7, respectively, p <0.001, Figure 12A, D, F), and in the middle area of the tongue (4±1 vs. 24.3±5.5, respectively, p <0.01, Figure 12A, E, G).

This suggests that *Trkb*$^{S/S}$ animals lose large proportion of neurons during gustatory development, which is likely reflected by the lower amount of available fibers innervating the taste buds present on the tongue. Whether this is also an issue with signalling remains to be seen.

As with previous developmental stages, we wanted to examine the possibility that the adaptor site mutation influences the amount of innervated taste buds present on the different parts of the tongue. We represented the amount of innervated taste buds on the tip as a proportion of total amount of innervated taste buds and found no difference between any of the genotypes: the amount of innervated taste buds in the tip area represented 82.6% in *Trkb*$^{W/W}$ mice, 81.8% in *Trkb*$^{P/P}$ mice and 76.7% in *Trkb*$^{D/D}$ mice (Figure 10C). From this we concluded that despite the fact that animals harbouring a mutation in the TrkB/PLCγ docking site had a higher amount of innervated taste buds present on the tongue than any other genotype, the innervation was consistent with the wild type control animals across the tongue regions. Similarly, the mice with the double mutation had a much lower amount of innervated taste buds present on the tongue, but this was consistently distributed across the tongue regions and the distribution was not altered.

On the other hand, animals bearing a mutation in the TrkB/Shc docking site were found to have 92.9% of all innervated taste buds present on the tip of the tongue, which is significantly higher than the wild type mice, where this area constitutes only 82.7% of all innervated taste buds (Figure 12C). This implies that the point mutation at the TrkB/Shc docking site causes loss of total number of innervated taste buds together with disruption of the conserved distribution of taste buds on the tongue seen in all the examined genotypes.

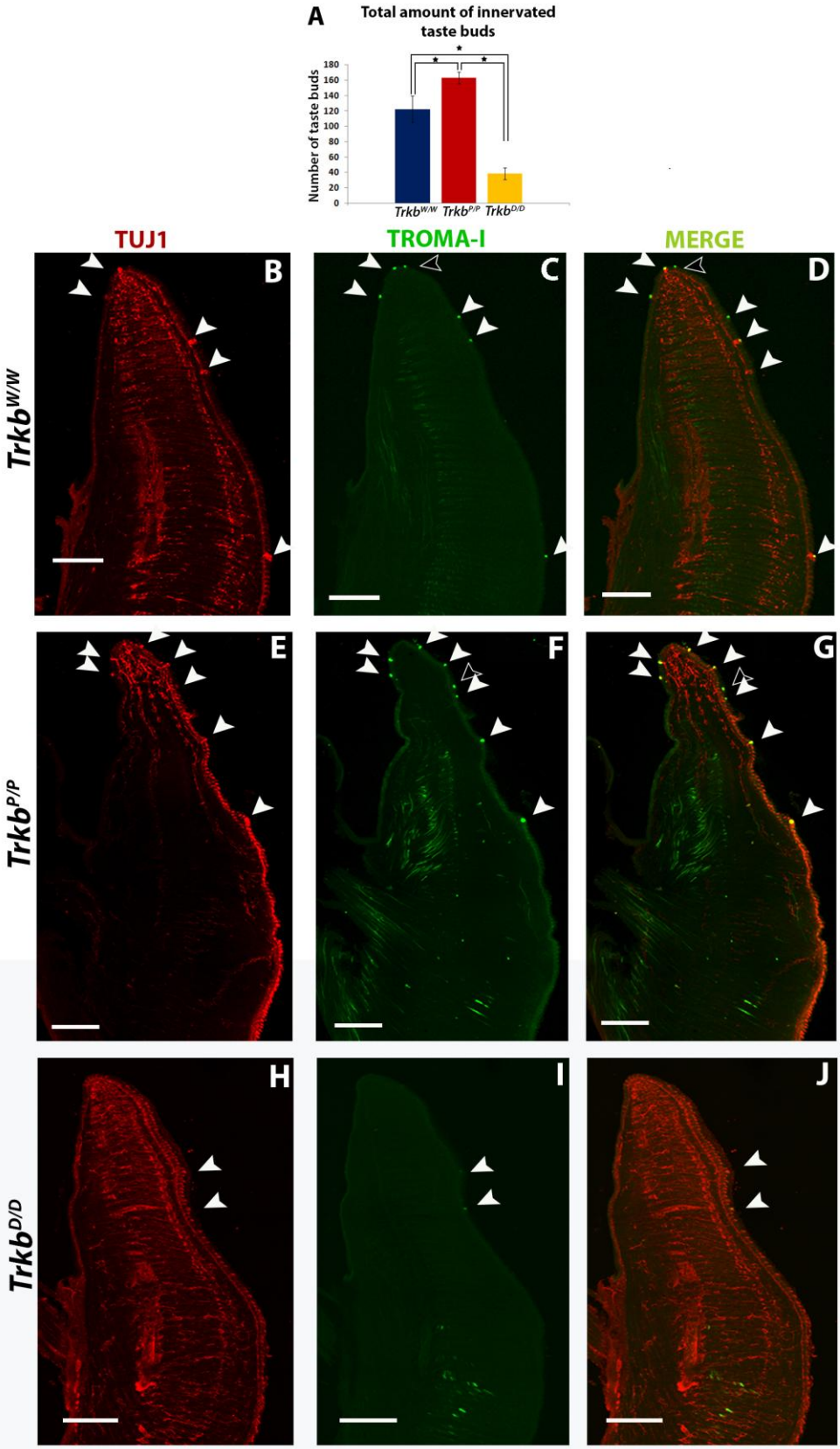
A
Total amount of innervated taste buds
Number of taste buds
180
160
140
120
100
80
60
40
20
0
Trkb^W/W
Trkb^P/P
Trkb^D/D
TUJ1
TROMA-I
MERGE
Trkb^W/W
Trkb^P/P
Trkb^D/D
B
C
D
E
F
G
H
I
J

Figure 9 (on previous page): Analysis of taste bud innervation at P0. **A**. *Trkb*$^{P/P}$ show higher number of innervated taste buds than *Trkb*$^{W/W}$ animals on the whole tongue, while *Trkb*$^{D/D}$ show a lower number of these than both animals **B-J** Representative sections of tongues stained by Tuj1 (**B**, **E, H**), Troma-I (**C**, **F, I**) and merged images of both (**D**, **G, J**). Arrowheads show the presence of innervated taste buds, empty arrowheads indicate uninnervated taste buds. All scale bars are 400µm. Significant difference was calculated using two-way ANOVA.

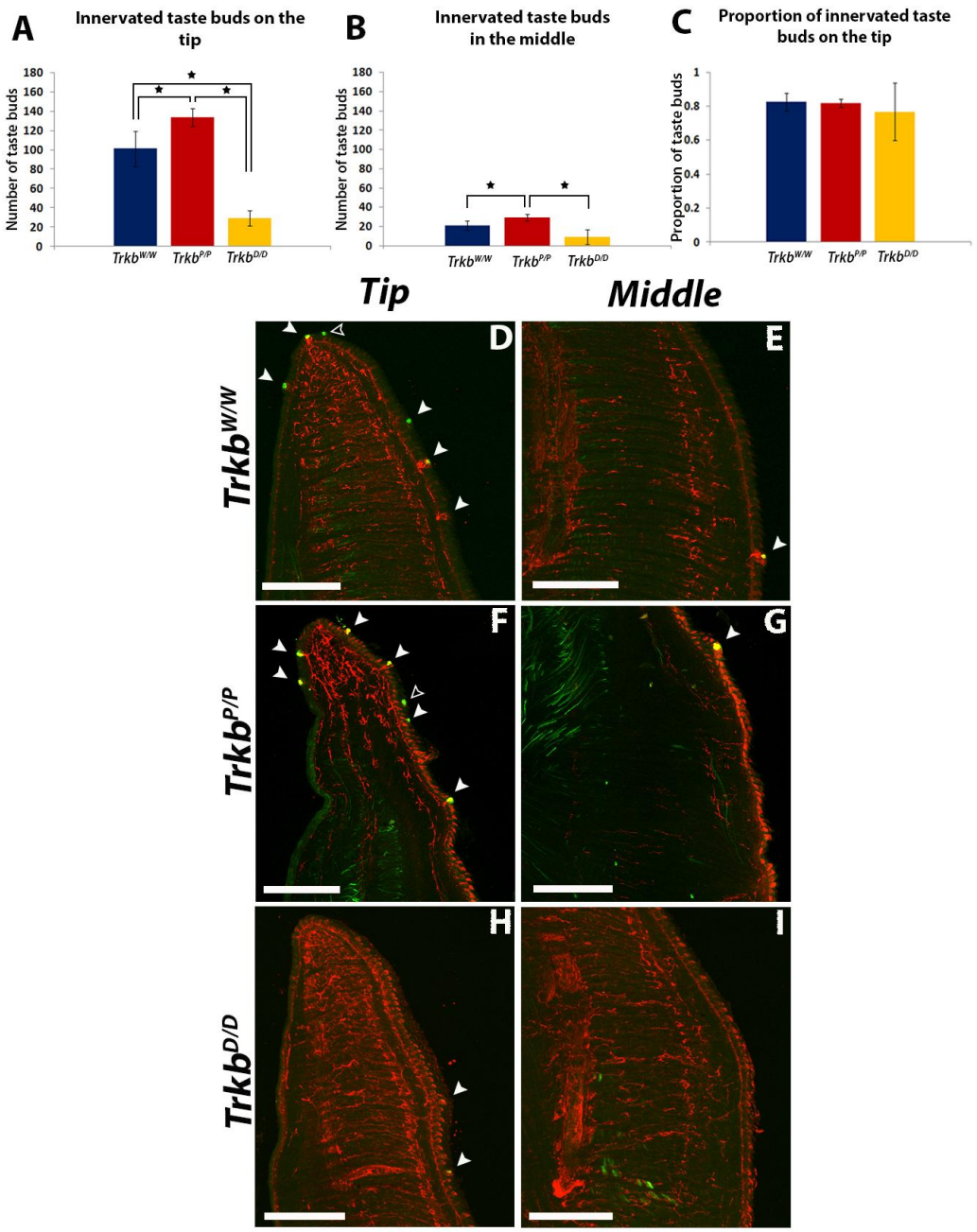

Figure 10: A. On the tip of the tongue *Trkb*$^{-/-}$ show higher number of innervated taste buds than *Trkb*$^{W/W}$ animals on the whole tongue, while *Trkb*$^{D/D}$ show a lower number of these than both animals. **B**. In the middle of the tongue, *Trkb*$^{P/P}$ animals have higher number of innervated taste buds than control animals, while *Trkb*$^{D/D}$ animals show no difference. **C**. Proportion of innervated neural buds on the tip of the tongue was not significantly different in any of the studied animals at P0. **D, F, H** show high magnification of tip of the tongue of and **C**,**E**,**G** high magnification of the middle of the tongue of *Trkb*$^{W/W}$, *Trkb*$^{P/P}$ and *Trkb*$^{D/D}$ animals in merged Tuj1 and Troma-I stain, respectively. Arrowheads indicate presence of taste buds; empty arrowhead show uninnervated taste buds. All scale bars are 400µm. Significant difference between proportions of taste buds was calculated using two-way ANOVA.

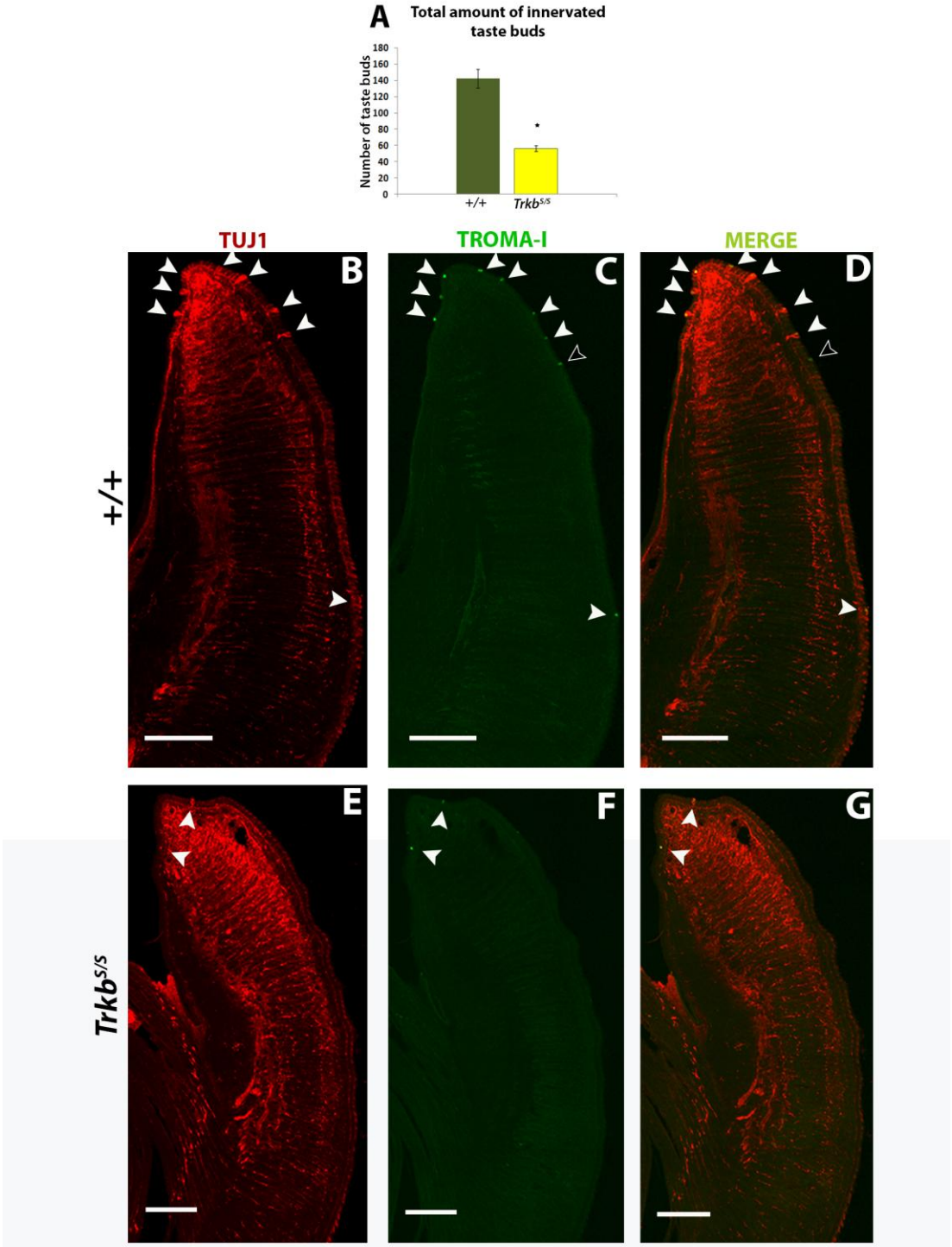

Figure 11: Analysis of taste bud innervation at P0 in animals with a point mutation in TrkB/Shc docking site. Animals carrying this point mutation showed a lower amount of innervated taste buds in the whole tongue, **A** when compared to control animals. **B-G** Representative images of tongue sections stained with Tuj1 (**B**, **E**), Troma-I (**C**, **F**) and merged images of both (**D**, **G**). Arrowheads show innervated taste buds, empty arrowheads indicate uninnervated taste buds. All scale bars are 400µm. Significant difference between the amount of innervated taste buds was calculated using Student's t-test.

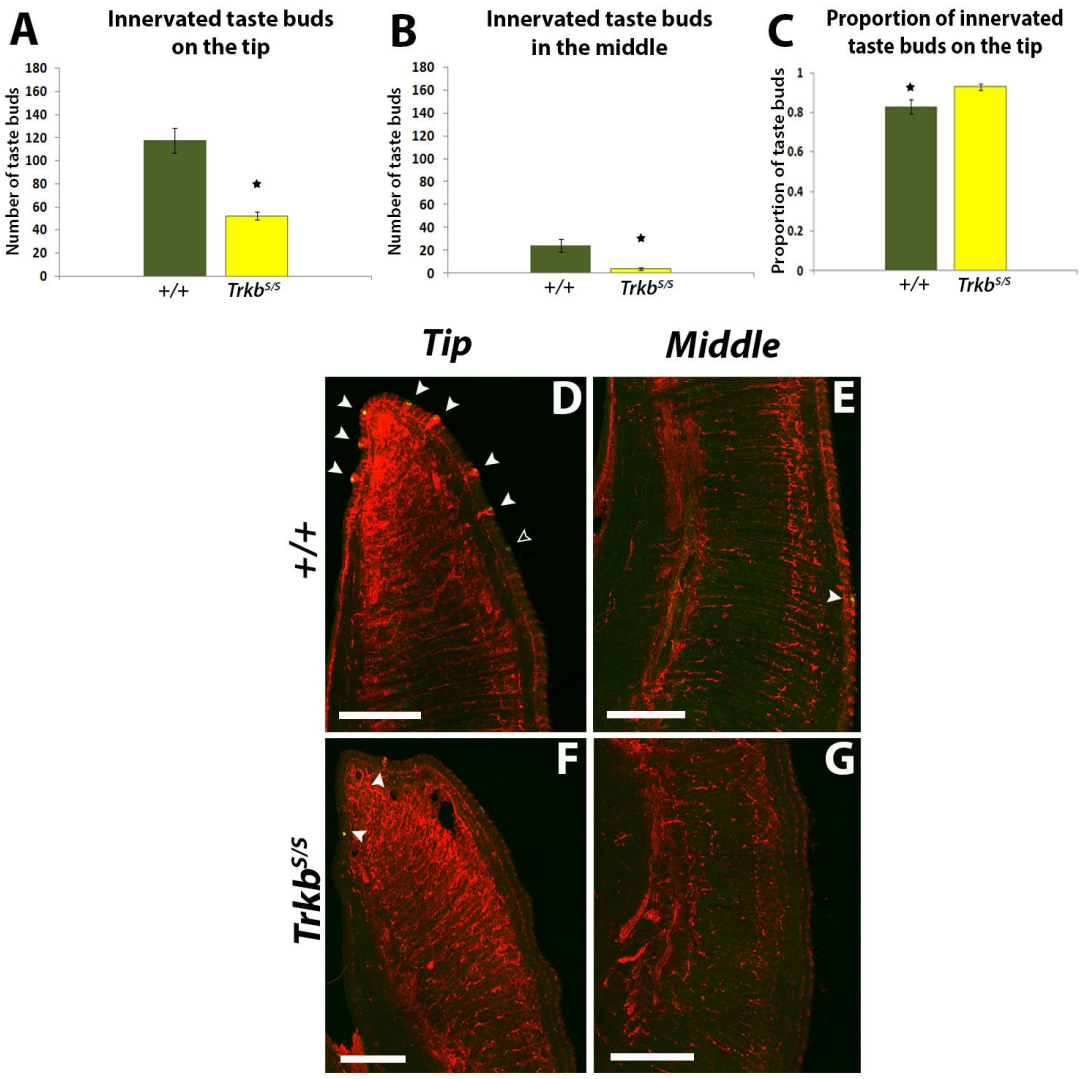

Figure 12: Taste buds of *Trkb*$^{S/S}$ animals show lower amount in the tip of the tongue (**A**), as well as in the middle (B), when compared to wild type controls. **C**. Proportion of innervated neural buds on the tip of the tongue was not significantly different in any of the studied animals at P0. **D, F** show high magnification of tip of the tongue of and **E**, **G** high magnification of the middle of the tongue of *+/+* and *Trkb*$^{S/S}$, respectively. All images represent merged staining of Tuj1 and Troma-I. Arrowheads indicate presence of taste buds; empty arrowhead show uninnervated taste buds. All scale bars are 400µm. Significant difference was calculated using Student's t-test.

3.2.3 Point Mutations in TrkB docking sites do not alter the innervation pattern to the tongue

At birth, as mentioned above, the development of the taste system is not yet complete, and the process of innervation of taste buds is still being modulated. Under normal circumstances a mature and functional taste bud is innervated by two to seven geniculate ganglion afferents (see Introduction), however, the innervation process is not finalized until a few days after birth. In order to determine whether disruption of neurotrophin signalling via point mutations in the TrkB docking sites influences this process we quantified the amount of uninnervated taste buds that are present on the tongue at P0. Because we used Troma-I antibody to examine the innervation of taste buds at this stage of development, we were able to identify taste buds that were not innervated and were instead represented as clusters of cytokeratin in the epithelial parts of the tongue.

We found that *Trkb*$^{W/W}$ mice contained 26±9.7 (n=6) uninnervated taste buds on the whole tongue, which was not significantly different from the *Trkb*$^{P/P}$ mice, whose tongues contained 19.9±10.26 (n=5, p=0.418) uninnervated taste buds. The *Trkb*$^{D/D}$ mice, on the other hand contained 12.7±12.2 (n=3) uninnervated taste buds on their whole tongues (Figure 13A). The number of uninnervated taste buds was not significantly different between either *Trkb*$^{D/D}$ and *Trkb*$^{W/W}$ (p=0.166), or *Trkb*$^{D/D}$ and *Trkb*$^{P/P}$ (p=0.509), presumably due to a large variability among the individuals within genotypes. Likewise, there was no significant difference in the proportion of uninnervated taste buds in either the tip or the middle part of the tongues (Figure 13B, C).

Trkb$^{S/S}$ mice, on the other hand, showed significantly lower number of uninnervated taste buds compared to the control animals (3.3±1.1, n=3 and 10.7±3.1, n=3, respectively, p=0.017), (Figure 13G, H, I), although due to low numbers of uninnervated taste buds found this difference was not observed in the middle area of the tongue.

These results suggest that the TrkB/PLCγ adaptor site mutation did not have an influence on the amount of uninnervated taste buds present on the tongue at P0, while point mutation at TrkB/Shc docking site caused a decrease in the amount of uninnervated taste buds at least on the tip of the tongue. However, due to the large variability of both innervated and uninnervated taste buds in all of the mutant animals we wanted to compare the amounts of innervated and uninnervated taste buds using a more informative method. We therefore standardized the total amount of (innervated and uninnervated) taste buds as 100% and calculated the proportion of innervated taste buds present in the different areas of the tongue. We found no difference between the *Trkb*$^{W/W}$, *Trkb*$^{P/P}$ and *Trkb*$^{D/D}$ mice for this aspect looking at either the whole tongue (0.81±0.06, 0.89±0.05, 0.79±0.16, respectively (comparison across all genotypes $p>0.15$), Figure 13D), tip part of the tongue (0.80±0.07, 0.89±0.06, 0.80±0.18, respectively, (all $p>0.13$), Figure 13E) or the middle part of the tongue (0.88±0.03, 0.89±0.13, 0.76±0.21, respectively (all $p>0.29$) Figure 13F).

Similar situation was found when we examined the proportion of innervated taste buds over the total amount of taste buds present on the *Trkb*$^{S/S}$ tongues and controls. Like the other genotypes we found that there was no difference in the proportion of innervated taste buds over the total on either whole tongues (0.93±0.02, 0.94±0.01, respectively, $p=0.405$, Figure 13J), tip part of the tongue (0.92±0.02, 0.95±0.04, respectively, $p=0.275$, Figure 13K), or the middle part of the tongue (0.97±0.05, 0.87±0.23, respectively, $p=0.499$, Figure 13L) between *Trkb*$^{S/S}$ and respective control mice. Despite finding a significant difference in absolute numbers between *Trkb*$^{S/S}$ and wild type mice, the proportional analysis results suggest that signalling via the TrkB/PLCγ and TrkB/Shc docking sites does not influence the proportion of innervated taste buds of all taste buds on the tongue.

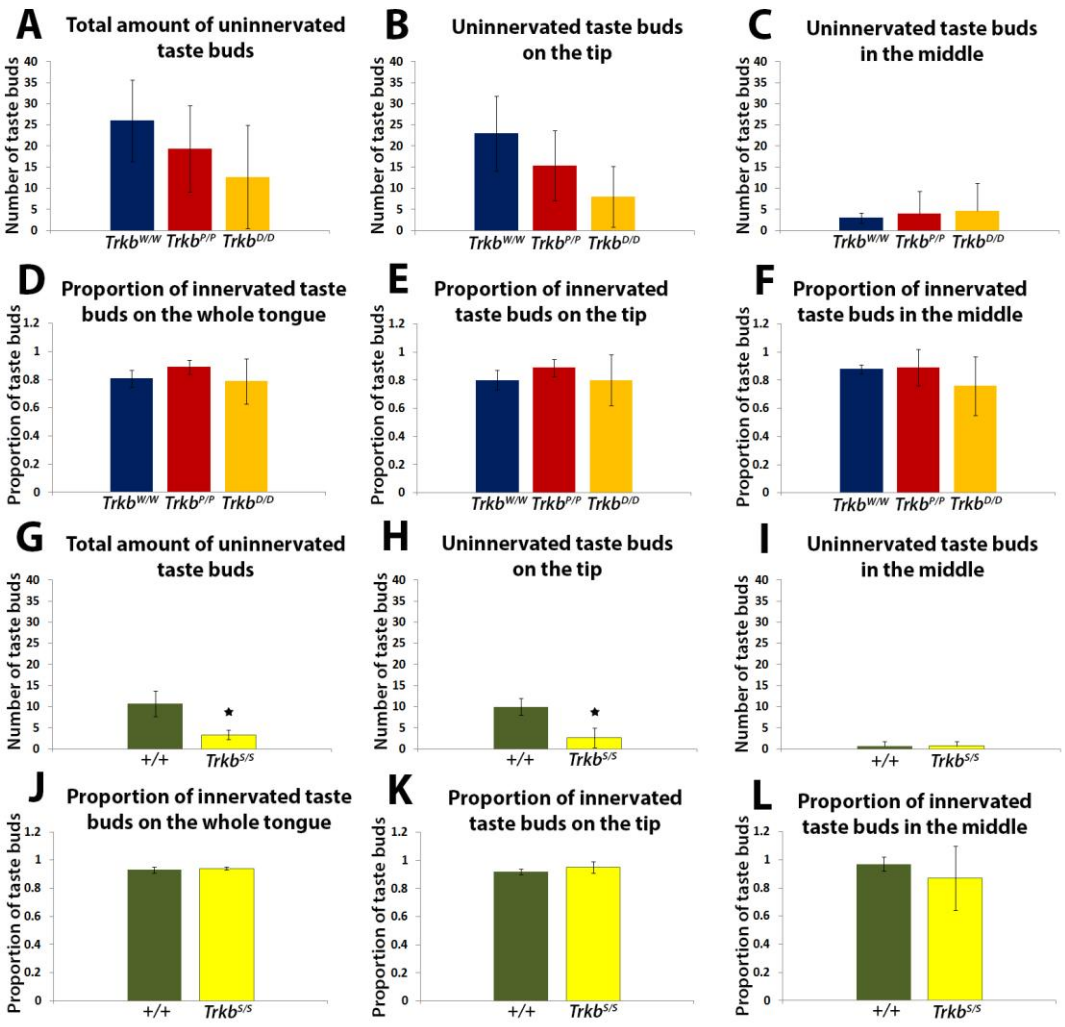

Figure 13: **A**-**C.** Comparison of uninnervated taste buds between genotypes at P0. The numbers of uninnervated taste buds were not significantly different between the *Trkb*$^{W/W}$, *Trkb*$^{P/P}$ and *Trkb*$^{D/D}$ animals on the whole tongue (**A**), tip of the tongue (**B**), and the middle of the tongue (**C**). **D-F**. Proportion of innervated taste buds in these animals was also not different in any area of the tongue analysed: whole tongue (**D)**, tip of the tongue (**E**), and middle of the tongue (**F**). **G-I**. The amount of uninnervated taste buds was significantly lower in TrkbS/S animals on the whole tongue (**G**), as well as on tip of the tongue (**H**), however, not in the middle of the tongue (**I**). **J-L**. Proportions of innervated taste buds were not different in any of the areas: whole tongue (**J**), tip of the tongue (**K**) and middle of the tongue (**L**). Significant difference was calculated using two-way ANOVA (**A-F**) and Student's t-test (**G-L**)

3.2.4 TrkB/Shc adaptor site influences innervation in adult mice while the TrkB/PLCγ site does not

In adult animals the taste system is fully developed, contains no or very few uninnervated taste buds, and is capable of detecting the full complement of tastes. In order to determine the effect of point mutations in TrkB receptor docking sites and to find out which, if any, signalling pathways determine the number of taste buds in a developed gustatory system, we analyzed the number of taste buds present in adult animals. Using the same analytical procedure as was used for examination of P0 animals, identifying individual taste buds using Tuj1 and Troma-I antibodies, we divided the tongue into three areas that were analyzed separately: the whole tongue (an area encompassing up to 8000µm from the tip of the tongue), the tip area of the tongue (up to 4000µm distance from the tip of the tongue) and the middle area of the tongue (4000-8000µm from the tip of the tongue) in adult animals (Figure 6C).

Trkb$^{P/P}$ mice were found to have not significantly different amount of innervated taste buds present on the whole area of the tongue when compared to the wild type control animals (108.3±28.5 vs. 90±13.1, respectively, n=3 for both genotypes, p=0.368, Figure 14A, B-G). There was no difference between these two genotypes in the tip area of the tongue (87±23.9 vs. 73±13.1, respectively, p=0.423, Figure 15A, D, F), or in the middle area of the tongue (21.3±6.4 vs. 20.3±5.8, respectively, p=0.850, Figure 15B, E, G).

On the other hand the *Trkb*$^{S/S}$ mice had significantly lower amount of innervated taste buds when compared to their wild type littermates considering the whole tongue (15±1 vs. 99.3±12.7, respectively, n=3 in both cases all p<0.001, Figure 16A, B-G), the tip area of the tongue (14±1 vs. 77.7±11.2, respectively, p<0.001, Figure 17A, D, F), as well as the middle part of the tongue (1±1 vs. 21.7±1.5, respectively, p<0.001), (Figure 17B, E, G).

These results suggest that despite there being a lack of innervation at E16.5 and recovery of innervation at P0 in the *Trkb*$^{P/P}$ animals, the innervation returns to normal state during adulthood. At this stage we weren't able to examine the

innervation in mice with mutation in both of their docking sites (*Trkb*$^{D/D}$) because these animals are not viable and do not survive into adulthood.

Mice carrying a point mutation in the TrkB/Shc docking site showed a decrease in innervation in adult stage, which is similar to the findings in P0 animals. No recovery in innervation is seen in these animals, which is likely due to the lack of geniculate ganglion neurons that was found in all the developmental stages in all of our experiments.

As with previous developmental stages we evaluated the representation of innervated taste buds on the different sections of the tongue. The taste buds on the tip area of the tongue represent 80.3% of all taste buds in *Trkb*$^{P/P}$ mice, which is not different from the proportion of taste buds in the tip area of the wild type control mice (80.8%, p=0.849) (Figure 15C). The proportion of innervated taste buds on the tip area of the tongue in *Trkb*$^{S/S}$ mice, however, is significantly different from those seen in their wild type littermates (93.4% vs. 78.1%, respectively, p=0.017), (Figure 17C). This would suggest that point mutation in TrkB/Shc docking site causes a disproportionate loss of taste buds present in the middle of the tongue compared to the wild type animals.

We evaluated the tongues of all the genotypes for the presence of uninnervated taste buds in the same way as we did with the tongues of P0 animals, however, the results in this case were drastically different. When we were looking for the uninnervated taste buds we found only one or two instances of an uninnervated taste bud in all the sections examined. We concluded that the reason for this may be that taste buds that are not innervated degenerate and would thus not be present on the tongue.

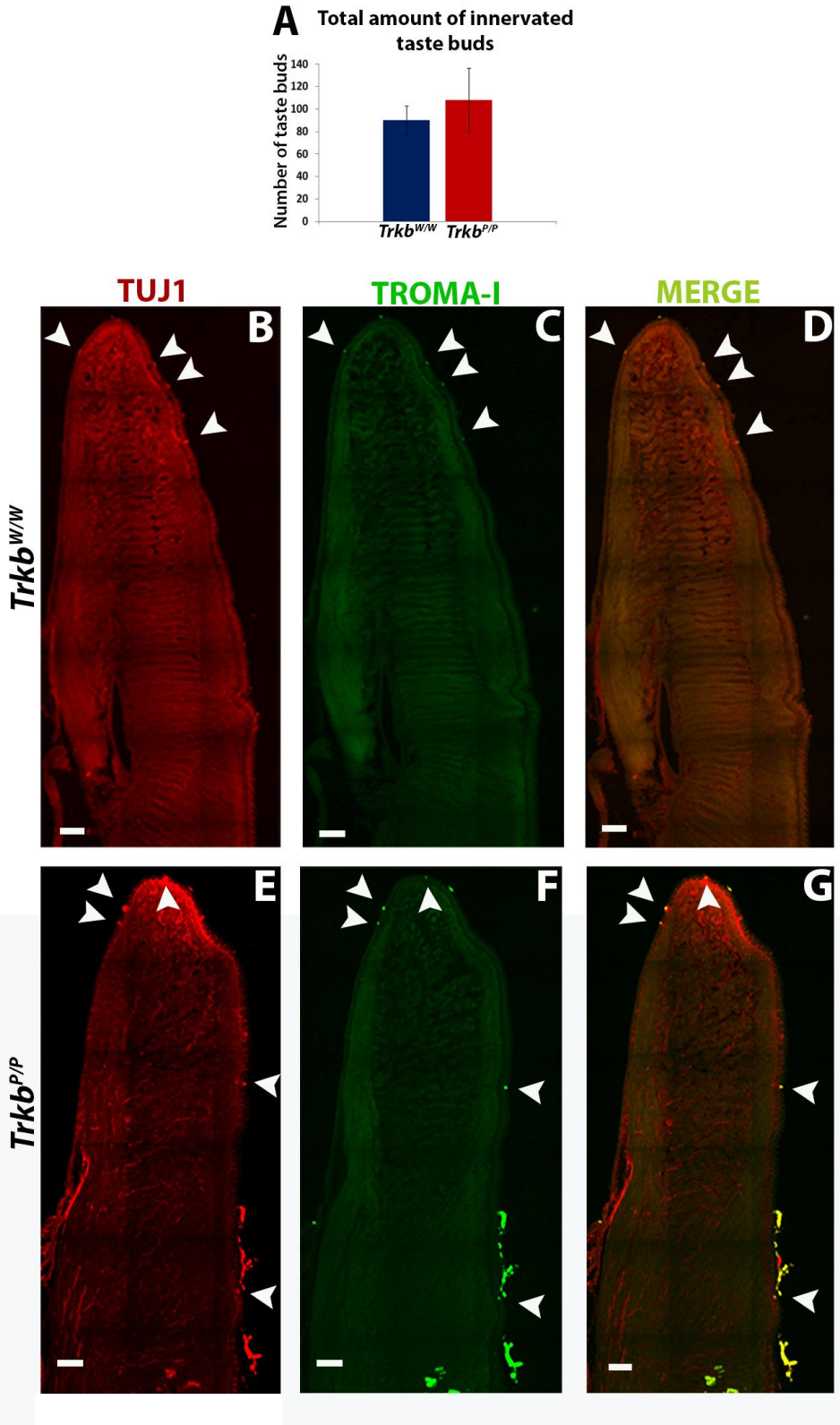

Figure 14: Analysis of taste bud innervation in adult animals. **A**. Amount of innervated taste buds present on the tongues of *Trkb*$^{W/W}$ and *Trkb*$^{P/P}$ mice is not different considering the whole tongue. **B-G** show representative tongue sections of *Trkb*$^{W/W}$ and *Trkb*$^{P/P}$ stained with Tuj1 (**B**, **E**), Troma-I (**C**, **F**) and merged images of both (**D**, **G**). Arrowheads indicate innervated taste buds. All scale bars are 400µm. Significant difference was calculated using Student's t-test.

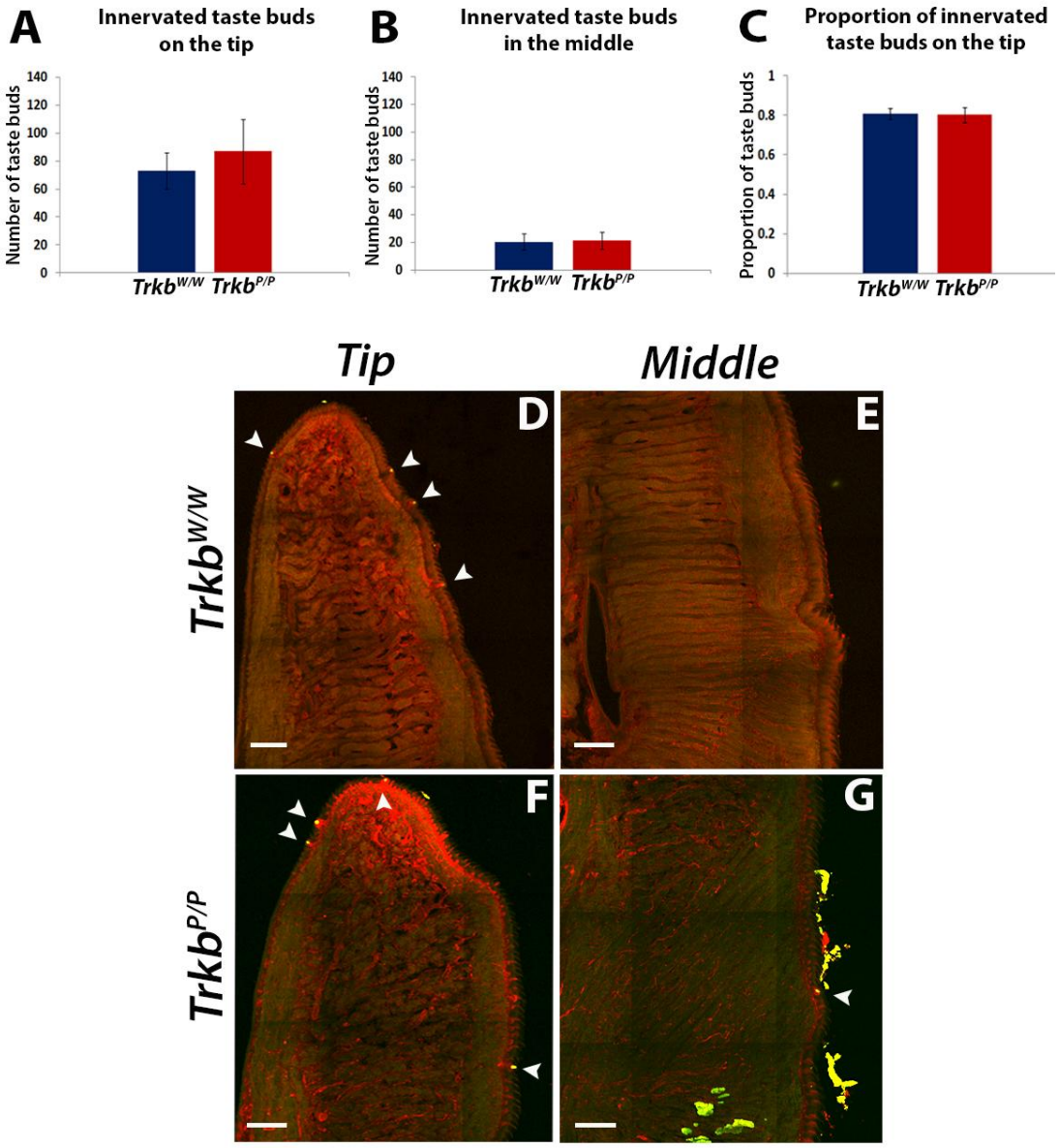

Figure 15: Anaysis of taste bud innervation in tongue partitions. The amount of innervated taste buds present in *Trkb*$^{P/P}$ was not significantly different from *Trkb*$^{W/W}$ animals in terms of either the tip of the tongue (**A**) or the middle of the tongue (**B**). **A.** Proportion of innervated neural buds on the tip of the tongue was not significantly different in any of the studied adult animals. **D, F** show high magnification of tip of the tongue and **E**, **G** high magnification of the middle of the tongue of *Trkb*$^{W/W}$ and *Trkb*$^{P/P}$, respectively. All images represent merged staining of Tuj1 and Troma-I. Arrowheads indicate presence of taste buds. All scale bars are 400µm. Significant difference between proportions of taste buds was calculated using Student's t-test.

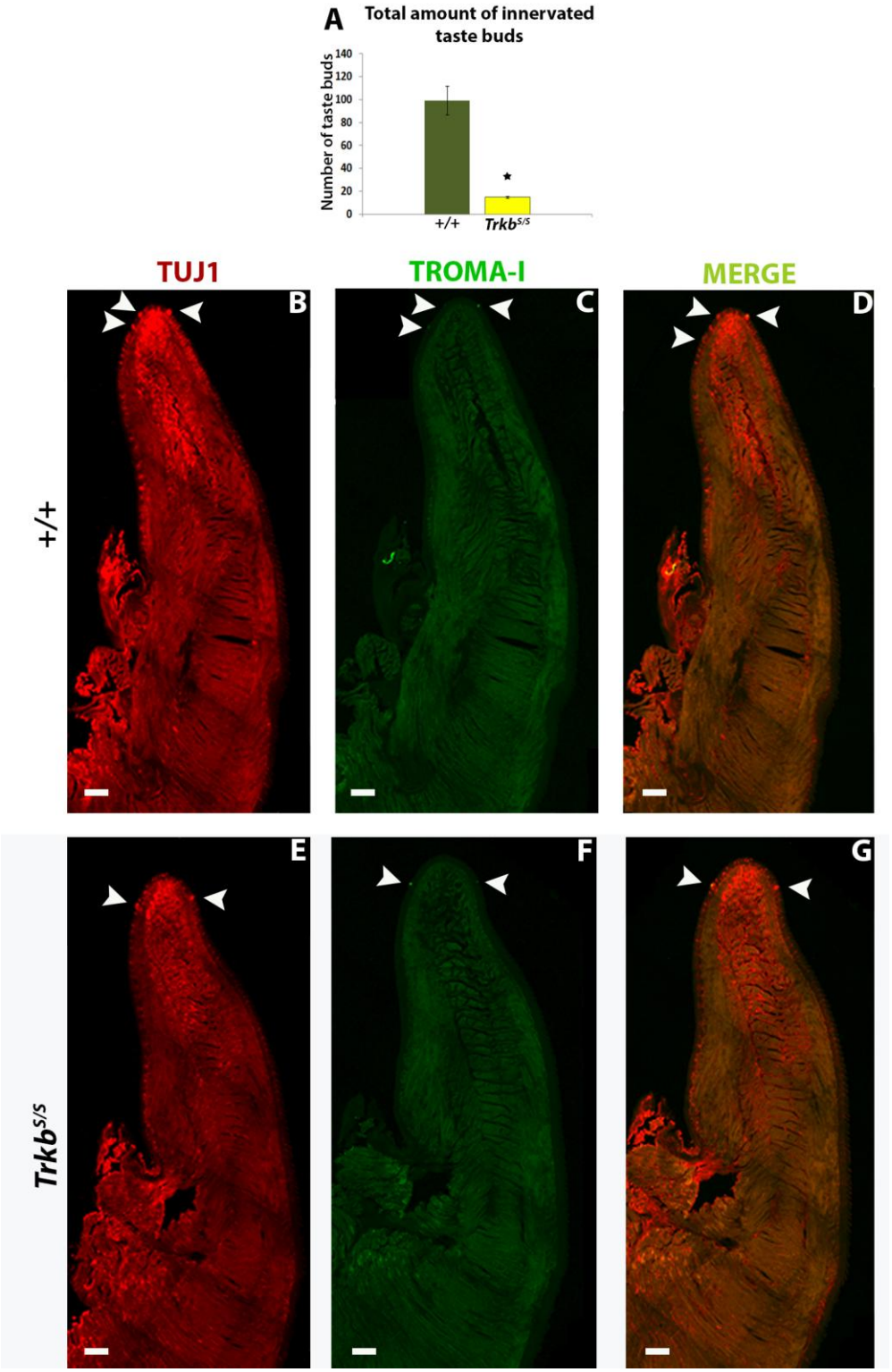

Figure 16: Analysis of taste bud innervation in adult animals with a point mutation in the TrkB/Shc docking site. **A.** Amount of innervated taste buds present on the tongues of these mice compared to wild type mice is lower considering the whole tongue. **B-G** Representative tongue sections of *+/+* and *Trkb*$^{S/S}$ mice stained with Tuj1 (**B**, **E**), Troma-I (**C**, **F**) and merged images of both (**D**, **G**). Arrowheads indicate innervated taste buds. All scale bars are 400µm. Significant difference was calculated using Student's t-test.

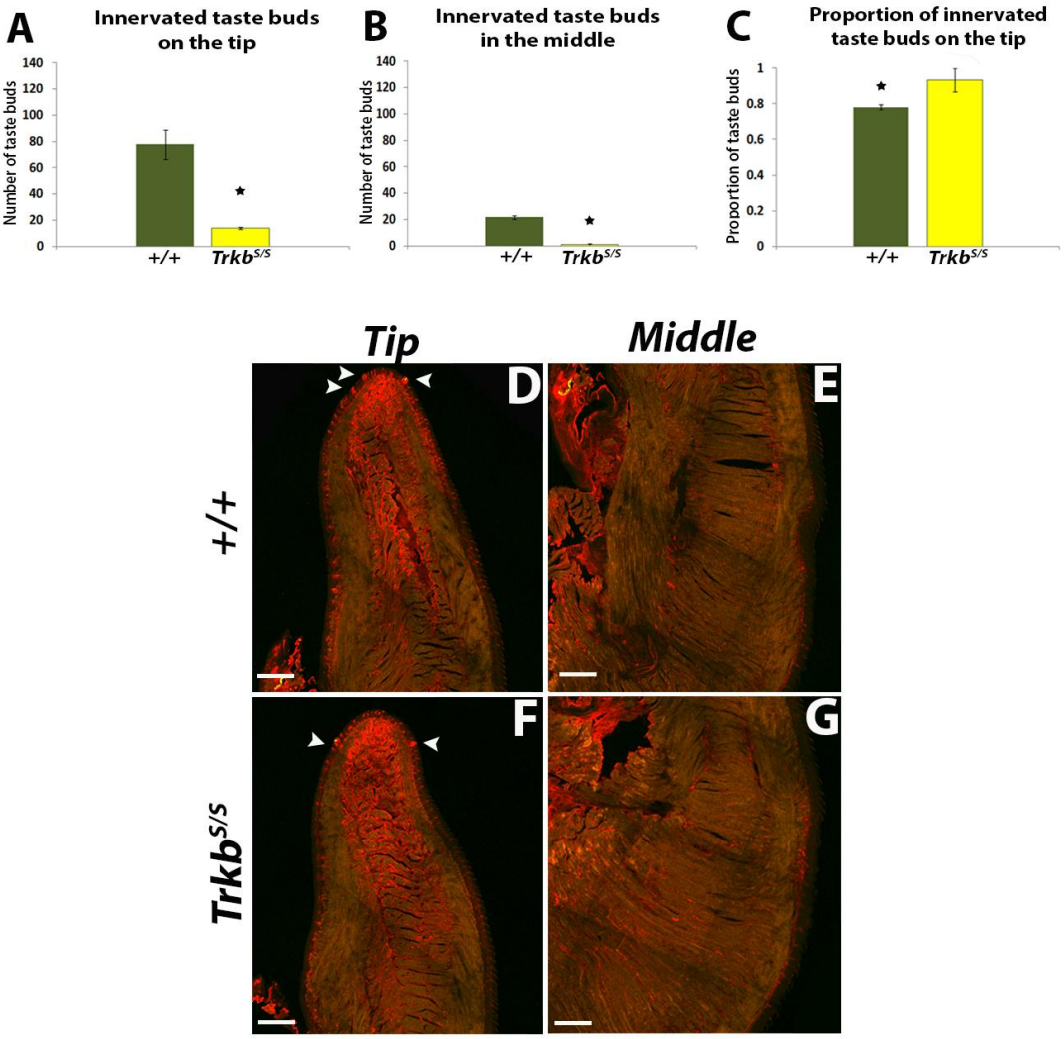

Figure 17: Analysis of innervated taste buds in the tongue partitions: The amount of taste buds present on tongues of TrkbS/S animals is lower than those of +/+ animals on the tip of the tongue (**A**) as well as in the middle (**B**). **C**. Proportion of innervated neural buds on the tip of the tongue was significantly higher in *Trkb*$^{S/S}$ animals when compared to their wild type counterparts. **D**, **F** show high magnification of tip of the tongue and **E**, **G** high magnification of the middle of the tongue of *+/+* and *Trkb*$^{S/S}$, respectively. All images represent merged stain of Tuj1 and Troma-I. Arrowheads indicate presence of taste buds. All scale bars are 400µm. Significant difference between proportions of taste buds was calculated using Student's t-test.

3.3 Morphological analysis of taste buds

3.3.1 Taste buds in P0 animals

Taste buds require two to seven geniculate ganglion axons in order to survive. This may influence the morphology of these taste buds given the variability of innervation in the different genotypes of mice analysed in these experiments. In order to determine whether the variable innervation into the tongue of the different mutant mice used in these experiments influenced the morphology of taste buds on their tongues, we examined the morphology of taste buds (in terms of their height and width, Figure 18N) in all of the different mutants and control mice based on cytokeratin staining using Troma-I antibody as a marker.

At birth, $Trkb^{P/P}$ taste buds were not significantly different from the wild type control taste buds, either in terms of their height or their width (Height (h), Width (w) $Trkb^{W/W}$ h: 23.57±3.56µm, w: 22.70±2.83µm n=3 (28 taste buds); $Trkb^{P/P}$ h: 22.26±2.13µm, w: 24.22±4.07µm n=3 (21 taste buds); height p=0.094, width p=0.129, Figure 18A, B, E-J). On the other hand, the $Trkb^{D/D}$ taste buds (h: 12.63±3.93µm, w: 12.22±2.53µm n=3 (14 taste buds), Figure 18A, B, K-M) were significantly smaller than the wild type control taste buds (height $p<0.001$, width $p<0.001$), as well as the $Trkb^{P/P}$ taste buds (height $p<0.001$, width $p<0.001$), likely reflecting the lack of innervation as seen by the loss of neurons in the geniculate ganglia during previous stages of development. This finding supports the fact that taste buds need innervation to properly develop and mature.

Previous experiments found that there may be differences in morphology of taste buds depending on their positioning on the tongue: Guagliardo et al. (2007) showed that taste buds on the middle part of the tongue degenerate faster after chorda tympani sectioning compared to the taste buds present on the tip part of the tongue. We therefore separated all the taste buds into taste buds present on the tip area of the tongue, and the ones present in the middle part of it, in a way that was identical to the separation of the tongue into different areas done for the

analysis of innervation at this stage. We have, however, found no differences in morphology between the tip part of the tongue or the middle part of the tongue and the sizes of taste buds in the whole tongues: taste buds present on the tip of the tongue of *Trkb*$^{W/W}$ were not significantly different from the *Trkb*$^{P/P}$ taste buds in terms of either height (p=0.110) or width (p=0.089); neither were those in the middle of the tongue (height p=0.649, width p=0.876). Taste buds on animals with the double mutation were smaller than the wild type control animals in all aspects in the tip part of the tongue (height p<0.01, width p<0.01). We were not able to analyze the taste buds in the middle of the tongue due to small sample size as only two taste buds were found (*Trkb*$^{W/W}$ tip h: 23.58±2.99µm, w: 22.92±2.88µm (24 taste buds), middle h: 23.54±3.56µm, w: 21.33±2.36µm (4 taste buds); *Trkb*$^{P/P}$ tip h: 22.21±2.02µm, w: 24.83±4.11µm (17 taste buds), middle h: 22.44±2.90µm w: 21.64±3.11µm (4 taste buds); *Trkb*$^{D/D}$ tip h: 13.98±4.13µm, w: 12.06±2.59µm (12 taste buds), middle h: 11.51±1.59µm, w: 13.97±1.75µm (2 taste buds), (Figure 18C, D).

Therefore, the animals with a point mutation in the TrkB/PLCγ docking site do not possess morphologically different taste buds when compared to the wild type controls. This is true when looking at the whole tongue or in any of the two areas of the tongue (the tip and the middle). On the other hand, a point mutation in both of the docking site, as in *Trkb*$^{D/D}$ animals, causes the taste buds to decrease in size, likely reflecting the lack of innervation into the taste buds, presumably due to the loss of geniculate ganglion neurons in all stages of development examined.

Examining the taste buds from P0 *Trkb*$^{S/S}$ we found that their taste buds were significantly smaller compared to their wild type littermates in terms of both of their height and width (*+/+* h: 19.58±3.55µm, w: 19.80±3.26µm, n=3 (24 taste buds), *Trkb*$^{S/S}$ h: 13.87±3.28µm, w: 13.88±4.49µm, n=3 (14 taste buds), height p<0.001, width p<0.001 (Figure 19A, B, E-J)).

Using the partition analysis of the tongue into the two areas, we found that the differences seen in this analysis were replicated when examining the whole tongue; the *Trkb*$^{S/S}$ taste buds were smaller than wild type littermate taste buds in

the tip area of the tongue in terms of both height and width (both $p<0.001$). We were not able to analyze the taste buds in the middle of the tongue due to small sample size as only two taste buds were found. *+/+* tip h: 19.09±3.14µm, w: 19.81±2.95µm (19 taste buds), middle h: 21.45±4.77µm, w: 19.75±4.68µm (5 taste buds); *Trkb*$^{S/S}$ tip h: 14.11±3.5µm, w: 13.46±4.74 µm (12 taste buds), middle h: 12.38±0.07µm, w: 16.41±0.15µm (2 taste buds), (Figure 19C, D).

The mutation in the TrkB/Shc docking site caused a decrease in the size of taste buds at P0 in the whole tongue and the tip of the tongue, although we were not able to evaluate the difference in the middle of the tongue. This suggests that the morphology of the taste buds in the tip of the tongue is affected by the loss of signalling via this adaptor site. Whether there is a difference between the morphology between the taste buds on the tip of the tongue and the middle of the tongue as shown by Guagliardo et al. (2007) remains to be seen.

Interestingly, the morphological analysis of taste buds in *Trkb*$^{S/S}$ and *Trkb*$^{D/D}$ revealed no significant difference in terms of either height ($p=0.861$) or width ($p=0.271$) on the whole tongue, tip (height $p=0.931$, width $p=0.379$) or middle of the tongue (height $p=0.522$, width $p=0.188$). This could reflect the high amount of geniculate ganglion neuron loss at all examined stages in both of these genotypes causing lower amount of geniculate fibers available to innervate taste buds on the tongue. Since the amount of innervation was previously implicated in directly influencing the size of taste buds (Krimm and Hill 1998) then, consequently, this could be the cause of the smaller sizes of taste buds in both *Trkb*$^{S/S}$ and *Trkb*$^{D/D}$ animals.

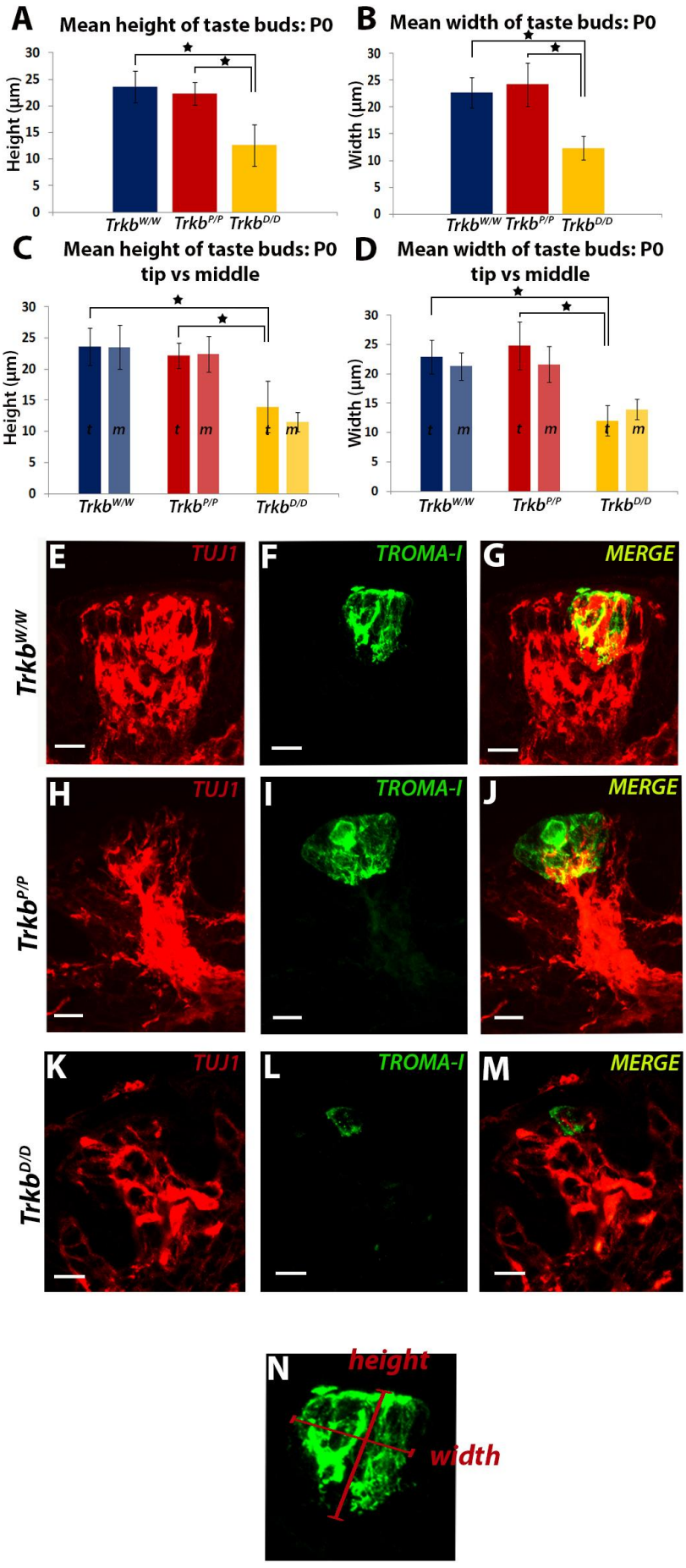
A
Mean height of taste buds: P0
Height (µm)
Trkb^W/W
Trkb^P/P
Trkb^D/D
B
Mean width of taste buds: P0
Width (µm)
C
Mean height of taste buds: P0
tip vs middle
D
Mean width of taste buds: P0
tip vs middle
t
m
E
TUJ1
F
TROMA-I
G
MERGE
H
I
J
K
L
M
N
height
width

Figure 18 (on previous page): Comparison of morphological attributes of innervated taste bud between *Trkb*$^{W/W}$, *Trkb*$^{P/P}$ and *Trkb*$^{D/D}$ mice at P0. **A**-**B** *Trkb*$^{P/P}$ taste buds are not different from wild type control taste buds in any aspect, *Trkb*$^{D/D}$ taste buds are lower in both height and width. **C**-**D.** The innervated taste buds of *Trkb*$^{D/D}$ animals show significantly smaller taste buds in terms of both height (**C**), and width (**D**) when compared to either *Trkb*$^{W/W}$ or *Trkb*$^{P/P}$ animals. Becaue of the small number of taste buds present in the middle of the tongue, the taste buds of *Trkb*$^{D/D}$ animals were not evaluated. **E-M** High magnification images of these taste buds: innervation is shown by Tuj1 (**E, H, K**), the taste bud by Troma-I (**F, I, L**) and the merged image of these two stains showing innervated taste buds (**G, J, M**). **N.** This panel depicts the standardised method used to measure the width and the height of the taste buds. All scale bars are 10µm. Significant difference was calculated using a two-way ANOVA.

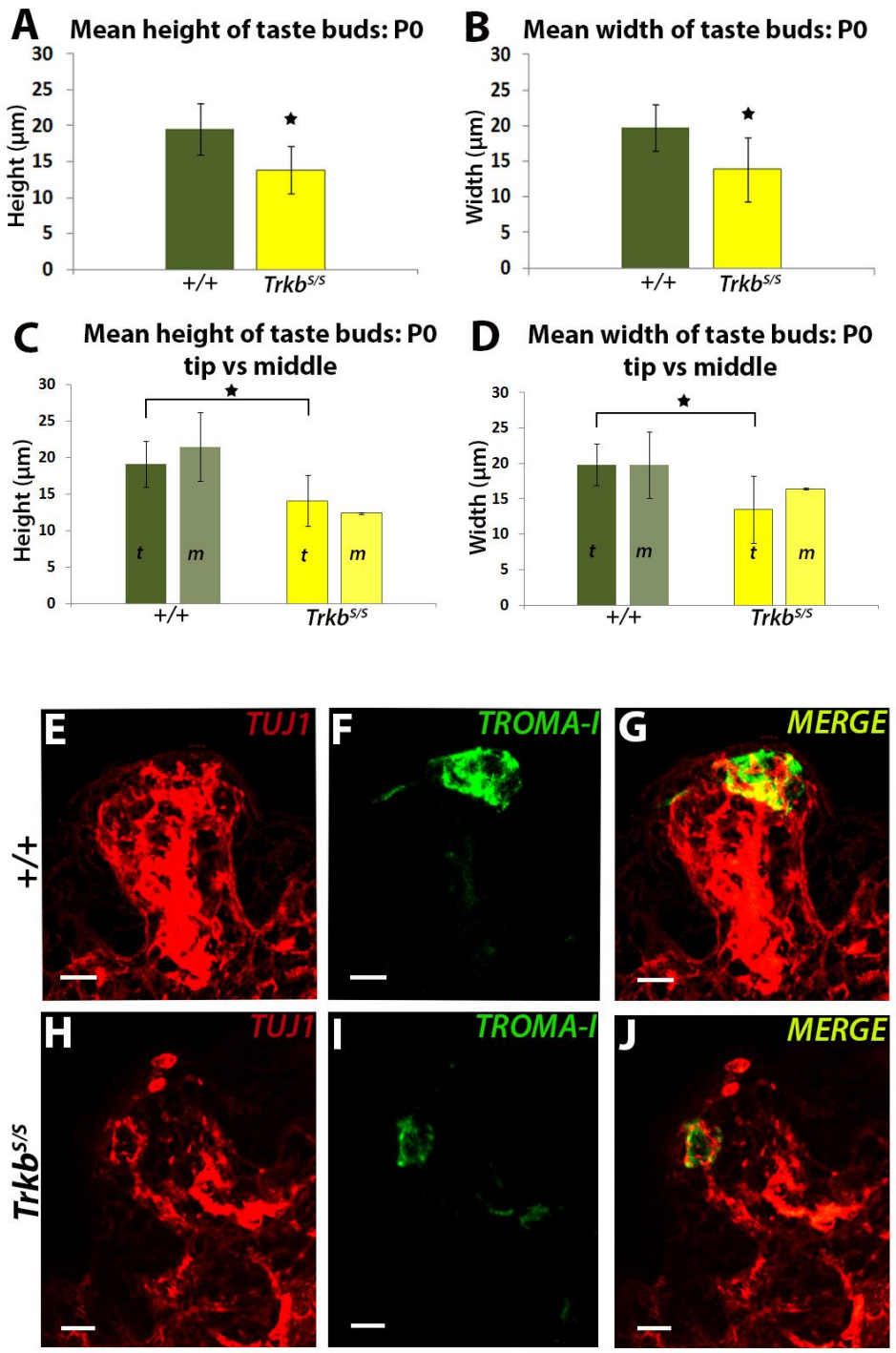

Figure 19: Morphological comparison of innervated taste buds between *+/+* and *Trkb*$^{S/S}$ mice at P0. **A-B** *Trkb*$^{S/S}$ taste buds were lower compared to wild type animals in terms of their height (**A**) and width (**B**). **C-D** The tips of the tongue of *Trkb*$^{S/S}$ animals were smaller than their wild type counterparts in both height (**C**) and width (**D**). On the other hand, no difference was found between the taste buds in the middle in terms of either height (**C**) or width (**D**). **E-J** High magnification images of taste buds; innervation is shown by Tuj1 (**E, H**), taste buds by Troma-I (**F, I**) and the innervated taste buds by merged images (**G, J**). All scale bars are 10µm. Significant difference was calculated using Student's t-test.

3.3.2 Taste buds on tongues of adult mice

As the development of the gustatory system in rodents is achieved after birth in adulthood, taste buds do not achieve full maturity until then. We wanted to determine whether the morphology of the taste bud differs between adult mice containing mutations in their adaptor sites. Examination of the morphological characteristics of taste buds in adult mice showed significant differences between taste buds present on tongues of *Trkb*$^{P/P}$ mutant and *Trkb*$^{W/W}$ control mice. The taste buds of *Trkb*$^{P/P}$ mice were significantly larger than their wild type controls in terms of height ($p<0.01$), however, the width difference between them wasn't significantly different ($p=0.732$) (*Trkb*$^{W/W}$ h: 38.20±8.25µm, w: 32.28±7.04µm n=2 (30 taste buds); *Trkb*$^{P/P}$ h: 42.85±6.22µm, w: 31.92±4.29µm n=2 (43 taste buds)), (Figure 20A, B, E-J).

Interestingly, the differences between the sizes of the taste buds were seen when looking at the tip of the tongue only: the taste buds present on the tip of the tongue were significantly taller compared to wild type control animals ($p=0.020$), but the width was not significantly different ($p=0.583$). In the middle part of the tongue we found no difference in the taste buds in terms of either their height (0.205) or width ($p=0.938$). *Trkb*$^{W/W}$ tip h: 37.57±8.13µm, w: 32.09±5.65µm (26 taste buds), middle h: 42.28±8.96µm, w: 33.50±14.49µm (4 taste buds); *Trkb*$^{P/P}$ tip h: 41.63±5.27µm, w: 31.41±4.12µm (36 taste buds), middle h: 49.08±7.37µm, w: 33.96±4.87µm (7 taste buds), (Figure 20C, D).

Analysis of *Trkb*$^{S/S}$ mutant taste buds, revealed significant differences compared to their wild type littermates in all of the aspects of the morphological analysis; the *Trkb*$^{S/S}$ taste buds were significantly larger in height compared to wild type animals ($p=0.014$), but smaller in width ($p=0.042$) (*+/+* h: 35.04±4.60µm, w: 33.69±4.13µm n=3 (33 taste buds), *Trkb*$^{S/S}$ h: 38.77±5.64µm, w: 30.98±4.72µm n=3 (17 taste buds)), (Figure 21A, B, E-J). Interestingly, when we examined the tip part of the tongues; the *Trkb*$^{S/S}$ taste buds were higher than wild type animals' taste buds ($p=0.013$), but we found no difference in width ($p=0.149$) (all $p>0.1$): *+/+* tip h:

34.62±4.28µm, w: 33.19±3.80µm (28 taste buds); *Trkb*$^{S/S}$ h: 38.47±5.50µm, w: 31.28±4.70µm (16 taste buds), (Figure 21C, D). Since we identified only one complete taste bud in the middle part of the *Trkb*$^{S/S}$ tongues, we could not statistically compare these to the numbers of the wild type animals.

We weren't able to examine the morphology of taste buds in *Trkb*$^{D/D}$ adult animals. Large difference in the taste bud morphology in P0 animals has been established between the double mutant animals and their wild type controls, but whether their taste buds remain different or they are recovered as seen in *Trkb*$^{S/S}$ animals is difficult to answer because *Trkb*$^{D/D}$ animals generally do not survive past the initial first few days of their lives, and so we can only hypothesize that seeing any recovery is unlikely.

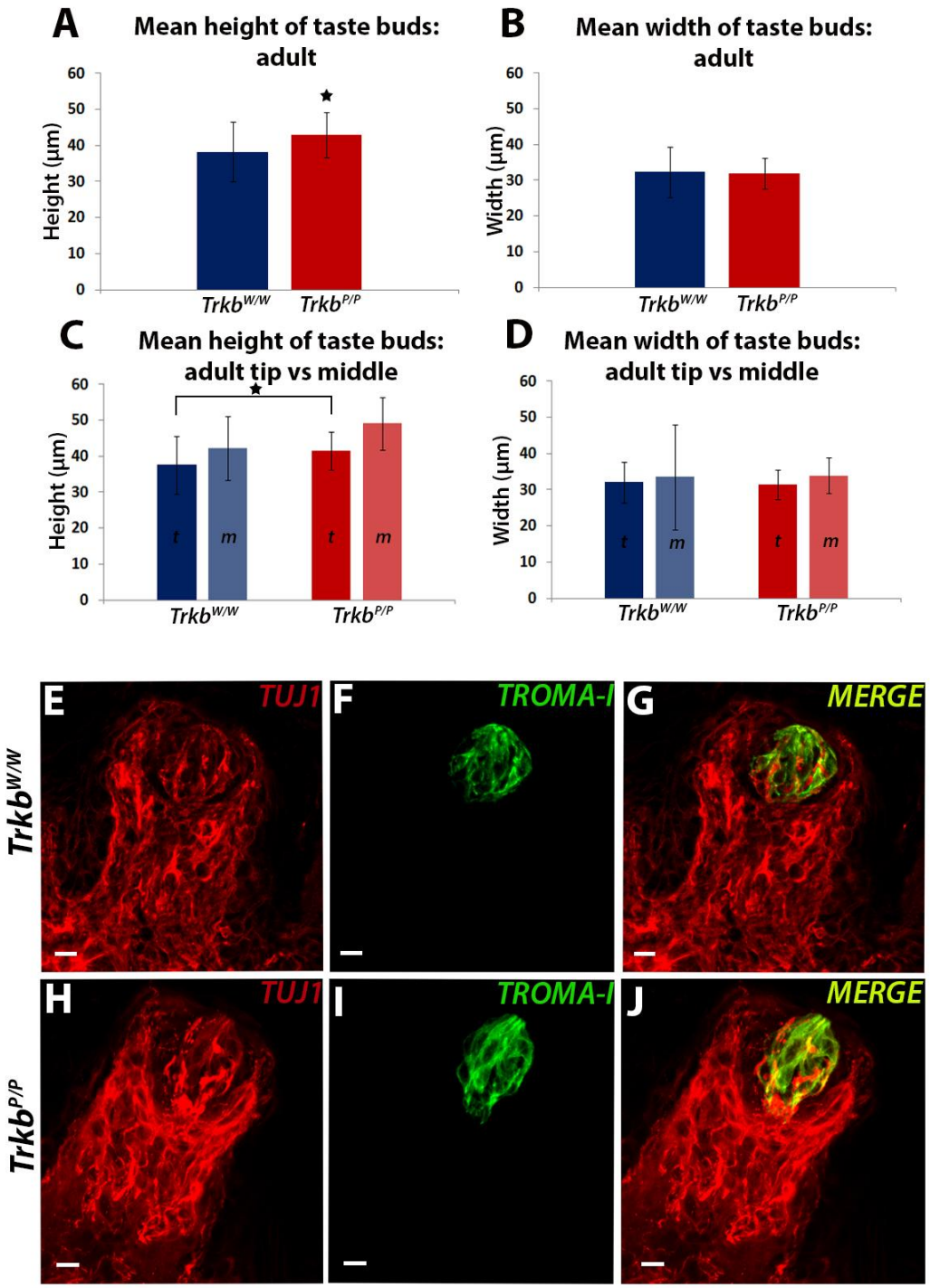

Figure 20: Morphological analysis of innervated taste buds present in *Trkb*$^{W/W}$ and *Trkb*$^{P/P}$ adult mice. **A-B** *Trkb*$^{P/P}$ taste buds are significantly higher than *Trkb*$^{W/W}$ taste buds in terms of their height (**A**), but not their width (**B**). **C-D** The taste buds present on the *Trkb*$^{P/P}$ mice were significantly taller only in the tip and not in the middle (**C**), while the division of the tongue revealed no difference in terms of width in either the tip or the middle (**D**). **E-J** High magnification images of taste buds; innervation is shown by Tuj1 (**E, H**), taste buds by Troma-I (**F, I**) and the innervated taste buds by merged images (**G, J**). All scale bars are 10µm. Significant diffence was calculated using Student's t-test.

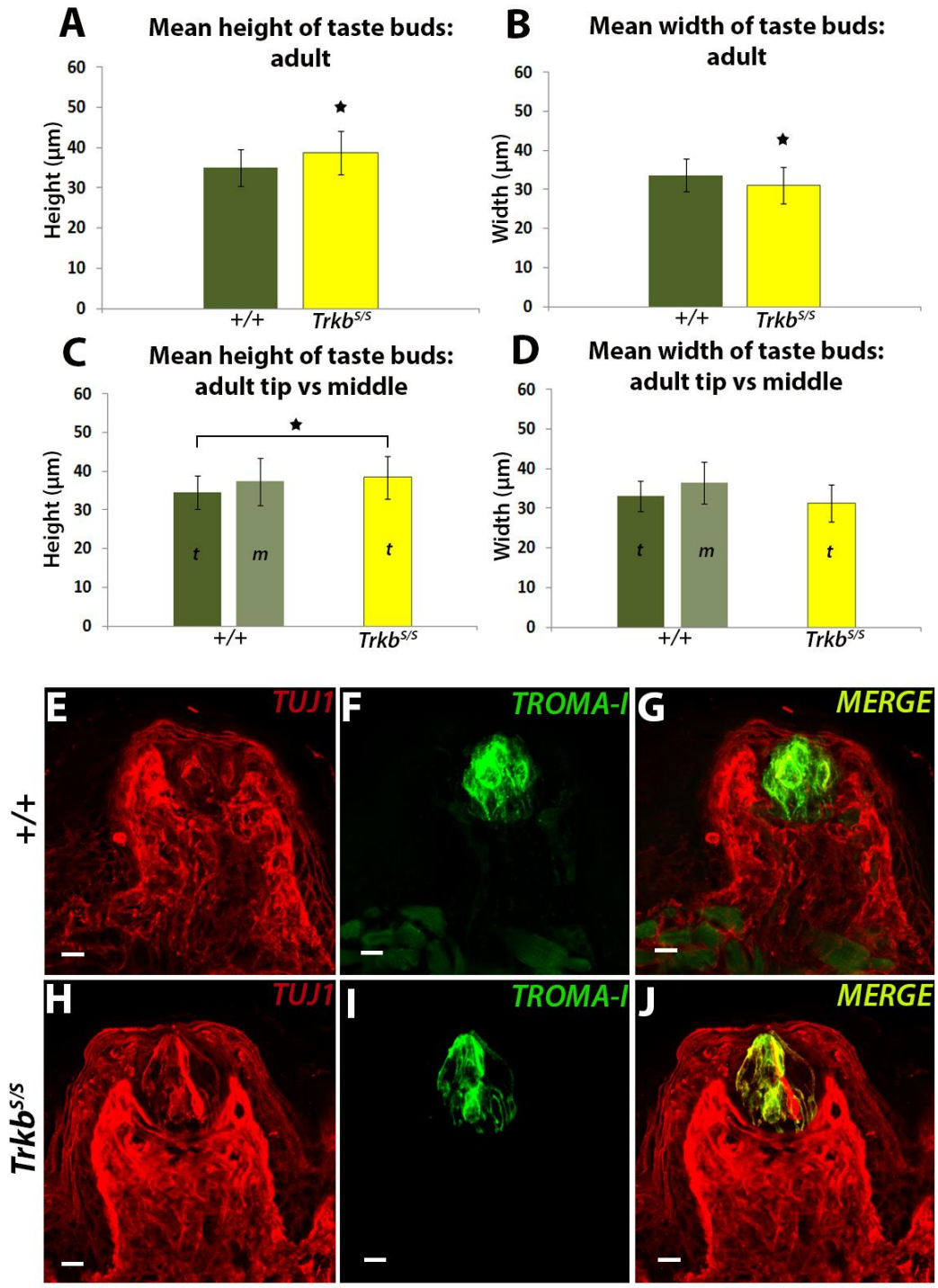

Figure 21: Morphological analysis of innervated taste buds in wild type and *Trkb*$^{S/S}$ mice at adult stage. **A-B** *Trkb*$^{S/S}$ innervated taste buds were higher than wild type innervated taste buds in height (**A**), but lower in width (**B**). **C-D** Significant difference was observed when looking at the tip of the tongue where the *Trkb*$^{S/S}$ innervated taste buds were larger than wild type taste buds in height (**C**), but not in width (**D**). **E-J**. High magnification images of taste buds; innervation is shown by Tuj1 (**E, H**), taste buds by Troma-I (**F, I**) and the innervated taste buds by merged images (**G, J**). All scale bars are 10µm. Significant difference was calculated using Student's t-test.

3.4 Results summary

We have found that the geniculate ganglion neuron survival is controlled by the signalling pathways downstream of the TrkB/Shc docking site, with the TrkB/PLCγ docking site playing only a minor role in this function. The TrkB/Shc docking site appears to mediate the effects of both BDNF and NT4 as the losses seen in animals with a mutation in this site show numbers of surviving geniculate ganglion analogous to those previously shown in *Bdnf* and *Nt4* null animals. The double mutation caused losses similar to previously shown in animals with double *Bdnf/Nt4* null and *Trkb* null mutations, thus demonstrating the crucial role of TrkB receptor in mediating the effect of neurotrophins BDNF and NT4 on geniculate ganglion neuron survival through the TrkB/Shc docking site.

We have also shown that the signalling pathways downstream of Trkb/Shc play an important role in innervation during the development of the gustatory system, however, we could not differentiate between the targeting and the loss of innervation due to previous loss of innervating geniculate ganglion neurons. On the other hand we determined that the TrkB/PLCγ docking site signalling pathways are crucial for guidance, and perhaps also the timing thereof, of geniculate fibers into the taste buds as we found delay of innervation during the development of the gustatory system, but also a return to normal state as the animals matured into adulthood. Loss of both docking site was shown to cause large losses in innervation throughout development once again showing the importance of TrkB downstream signalling in the rodent gustatory system.

Finally, we have shown that the signalling pathways mediated by these two docking sites are also able to influence the morphology of taste buds. Signalling pathways downstream of TrkB/Shc docking site appear to influence the morphology of taste buds early after birth, as well as in the adulthood, while the signalling pathways downstream of TrkB/PLCγ site exert their influence primarily in adult animals. This could also reflect the delayed developmental growth as demonstrated by innervation patterns in animals with mutation in this docking site.

Overall, we have demonstrated that the two docking sites present on the TrkB receptor are both important for various aspects of the rodent gustatory development, and while being activated by the same ligands they have different roles during this process.

4. DISCUSSION

4.1 Geniculate ganglion neuron survival

4.1.1 Geniculate ganglion survival during gustatory development is influenced by a point mutation in TrkB/Shc docking site

Neurotrophins signalling via the Trk receptors have been found to play an important role in determining the survival of neurons in ganglia of the peripheral nervous system, namely the sensory neurons in the dorsal root, vestibular and cochlear, trigeminal, geniculate and the nodose-petrosal ganglia. This thesis is focused on the ganglion of the rodent gustatory system, the geniculate ganglion. The role of neurotrophins in this ganglion has been analyzed previously: both BDNF and NT4 act via TrkB to influence the survival of the geniculate ganglion neurons during development. *Nt4* null mice lose about half of the geniculate ganglion neurons early in gustatory development around embryonic day 12.5, while *Bdnf* null mice lose the same amount of neurons two days later, at E14.5 (Patel et al. 2010a, Patel et al. 2012). Experiments with double *Bdnf/Nt4* null mice as well as with *Trkb* null mice showed that vast majority of geniculate ganglion neurons was lost during embryonic stages of these mice (Conover et al. 1995, Fritzch et al. 1997). It is unclear how the neurotrophins function uniquely through the same receptor. Since the affinities of the neurotrophins to the TrkB receptor are not different (Barbacid 1995, Minichiello et al. 1998), it is unlikely that this would be the cause of influencing the differential ability of these to determine the geniculate ganglion neurons survival. It is, however, possible that one or both of the neurotrophins are involved in activation of, or cross-talk with, the p75NTR (See Introduction 1.1.2. and discussed below), as losses of geniculate ganglion neurons have been reported in *p75* null mice (Krimm 2006). This could explain why the neurotrophins are able to influence survival of different populations of neurons in the geniculate ganglion. Whether this is the case remains to be seen.

We sought to determine whether the different outcomes of TrkB activation by these two neurotrophins can be explained by different signalling pathways

downstream of this receptor. Specifically, examining the influence of point mutation at two specific docking sites, TrkB/PLCγ and TrkB/Shc, would establish whether BDNF and NT4 can activate different signalling pathways despite acting on the same receptor. To this end a total of three stages were examined, E12.5, E14.5 and P4 in order to encompass all of the stages of neurotrophin influence during development, as well as capture the time prior to and post- innervation into the tongue (E14.5 in mice).

TrkB/Shc docking site was found to have a profound influence on the neurons in the geniculate ganglion. Mice with a point mutation in this site lost a large proportion of their neurons throughout development, starting at E12.5, where the loss was shown to be 20% of all geniculate ganglion neurons. This effect was exacerbated with development, as at E14.5 the loss increased to 66% while by P4, a stage at which the development of the geniculate ganglion is considered to be complete, the loss of neurons reached 73%. Interestingly, examining the geniculate ganglion during the gustatory development of embryonic and P4 mice, we found no change in the number of neurons in mice with a point mutation in the TrkB/PLCγ docking site at any stage analysed during development. Examination of animals with the double docking site mutation (*Trkb*$^{D/D}$), on the other hand, revealed a large loss of neurons throughout development, starting at E12.5 where 80% of geniculate ganglion neurons were lost compared to the wild type control animals. The survival of geniculate ganglion neurons worsened in older embryos, at E14.5, when the loss of these neurons reached 90%. We confirmed that TrkB receptor is the principal factor whose signalling determines the survival of the geniculate ganglion during embryonic development. We also showed that downstream signalling of BDNF and NT4 is facilitated by this receptor, as the double mutant *Bdnf/Nt4* animals lost the same amount of neurons as did *Trkb*$^{D/D}$ animals and animals with *Trkb* null mutation (Liu et al. 1995, Conover et al. 1995, Fritzsch et al. 1997).

4.1.2 TrkB/Shc facilitates signalling of NT4 in early development of neurons in the geniculate ganglion

Our results strongly suggest that TrkB/Shc adaptor site facilitates the neurotrophin signalling during gustatory development, which influences the survival of the geniculate ganglion. The TrkB/PLCγ docking site, on the other hand, may be involved in an indirect way as no neuronal losses were observed in *Trkb*$^{P/P}$ animals.

It has been shown previously that NT4 exerts an influence on the survival of the geniculate ganglion neurons early in development, at E12.5, as half of the neuronal population of geniculate ganglion neurons is lost in *Nt4* null mutant embryos (Patel et al. 2012). We found that *Trkb*$^{S/S}$ mice lost 20% of their neurons at this stage. Since TrkB/PLCγ point mutant animals did not lose any geniculate ganglion neurons at this stage of development, we concluded that TrkB/Shc docking site facilitates the influence of NT4 signalling on survival. *Trkb*$^{D/D}$ animals lost 80% of their geniculate ganglion neurons at E12.5. This is comparable to what has been found by Fritzsch et al. (1997), who showed that *Trkb* null mice lost about 70% of the geniculate ganglion neurons at the same stage, highlighting the importance of TrkB downstream signalling mediated by these two docking sites.

Because the double mutant animals lost a much larger proportion of neurons in the geniculate ganglion than animals with a mutation in only the TrkB/Shc docking site, it is likely that the intact TrkB/PLCγ docking site plays a supportive role that allows a proportion of neurons in this ganglion to survive despite the lack of TrkB/Shc. It has been shown previously that the TrkB/PLCγ site is able to associate itself with GAB1, a protein that is important in signalling pathways downstream of the TrkB/Shc docking site (Minichiello 2009, Medina et al. 2004). This means that when the TrkB/Shc docking site is dysfunctional, as it is in Trkb$^{S/S}$ animals, a proportion of the signalling that influences survival of the geniculate ganglion neurons may be compensated by signalling via the TrkB/PLCγ site. This also explains why the loss in these animals is less severe when compared to animals with *Nt4* null mutation. In *Trkb*$^{D/D}$ and in *Trkb* null mice, however, none of the two sites

are present, which means that TrkB/PLCγ site is not able to provide compensation of signallling and thus the losses of geniculate ganglion neurons are more severe.

Furthermore, results from these experiments imply that there may be a proportion of neurons in the geniculate ganglion that are dependeng on survival signalling via either the TrkB/Shc or the TrkB/PLCγ docking site, but there are no neurons that are dependent on signalling via the TrkB/PLCγ site only.

Another possibility explaining the loss of geniculate ganglion neurons in *Trkb*$^{S/S}$ animals is that signalling pathways downstream of the TrkB/Shc docking site may be triggered by a site present at a separate region of the TrkB receptor. By binding directly to the phosphotyrosine residues in the catalytic domain of the TrkB receptor, Src homology domain-containing proteins SH2B and SH2B2 are able to recruit GRB2, which is involved in signalling pathway downstream of the TrkB/Shc docking site (See Introduction, Qian 1998, Minichiello 2009). It is therefore possible that in the event of point mutation in the TrkB/Shc docking site, some of the signalling is substituted by the secondary docking site via SH2B and SH2B2, thus causing the loss of geniculate ganglion to be less severe compared to *Nt4* null mutant animals, where the survival of these neurons is compromised.

The reason we observe a large loss of geniculate ganglion neurons in *Trkb*$^{D/D}$ animals may be an additive effect in these animals. Sciaretta et al. 2010 showed that *Trkb*$^{D/D}$ mice lost large proportion of the vestibular sensory neurons, which was shown to be equal to one seen in *Trkb* null mutant animals (Minichiello 1995). Furthermore, these mice were shown to have diminished cortical neuronal migration in the central nervous system, and a much shorter lifespan than normal mice (Medina et al. 2004). Losing signalling downstream of TrkB receptor therefore has a profound and crucial influence on the whole organism. The reason such a large proportion of geniculate ganglion neurons are lost in these animals could therefore be the loss of TrkB signalling in the whole system causing large amounts of damage, rather than losses of supportive roles of other docking sites.

4.1.3 TrkB/Shc facilitates the signalling of both NT4 and BDNF during later geniculate ganglion development

At E14.5 we found that the loss of geniculate ganglion neurons in the TrkB/Shc mutant animals increased to 66%, while the TrkB/PLCγ point mutant animals lost no geniculate ganglion neurons. The loss of these neurons in *Trkb*$^{D/D}$ animals was 90%.

At this stage of development, the *Nt4* null mutant mice and the *Bdnf* null mutant mice lost half of the geniculate ganglion neurons (Patel et al. 2010, Patel et al. 2012), while animals with *Trkb* null mutation lost geniculate ganglion proportion very similar to *Trkb*$^{D/D}$ mice, nearly 90% (Fritzsch et al. 1997) (animals with a double *Bdnf/Nt4* null mutation have not yet been examined in early development). At E14.5 the geniculate ganglion neuron survival is therefore influenced by both TrkB receptor ligands, BDNF and NT4, while Trkb/Shc docking site is still the only site capable of facilitating the signalling for this process directly. Same as with earlier developmental stages, a point mutation in the TrkB/PLCγ docking site alone does not cause loss of geniculate ganglion neurons. We therefore sought to determine which signalling pathways regulate the geniculate ganglion neuron survival at this stage of development.

Both neurotrophin-mutant animals have been shown to lose only about half of the geniculate ganglion neurons, while the TrkB/Shc point mutant animals lost 66%. This suggests that the TrkB/Shc docking site is able to facilitate the survival signalling from both neurotrophins, while not being the single docking site influencing the survival; and the TrkB/PLCγ docking site may be able to compensate for any further loss of neurons. This implies that while some neurons in the geniculate ganglion are dependent on signalling via TrkB/Shc docking site as well as signalling via both docking sites at the same time, there are no neurons that are dependent solely on TrkB/PLCγ docking site in terms of their survival.

4.1.4 TrkB/PLCγ docking site could support survival of a third of geniculate ganglion neurons

Examining the possibility of a supporting role of TrkB/PLCγ reveals interesting facts about surviving neurons. At E12.5, animals with a point mutation in the TrkB/Shc docking site lose 20% of geniculate ganglion neurons, while *Nt4* null mutant mice lose 50%. We can hypothesize that TrkB/PLCγ site supports about 30% of neurons in case of loss of signalling through the TrkB/Shc site. At E14.5, TrkB/Shc point mutation causes a loss of 66% of geniculate ganglion neurons. At this stage, both neurotrophins are able to influence the survival of geniculate ganglion neurons by 50% each. Since both the double mutant animals $Trkb^{D/D}$ and *Trkb* null animals lose most of their geniculate ganglion neurons, we can also hypothesize that *Bdnf/Nt4* double null mutants would lose most of these neurons as well (experiments with *Bdnf/Nt4* double mutation in young embryonic mice have not been done, although these animals were shown to lose a large proportion of the geniculate ganglion neurons prior to birth (Conover et al. 1995, Liu et al. 1995)). In this case animals with a loss of the only docking site able to support neuron survival, TrkB/Shc, would normally lose about 90% of geniculate neurons, but since the TrkB/PLCγ docking site substitutes the TrkB/Shc docking site for about 30% of the geniculate ganglion neurons, the loss of neurons is not as severe. Whether this is done by substituting a part of the downstream signalling pathways of the TrkB/Shc docking site or by compensatory signalling via this docking site remains a question.

Interestingly, the loss of geniculate ganglion neurons in the animals with point mutation in the TrkB/Shc docking site is increasing with developmental progress until E14.5, which leads to a loss of 66%. At this point the loss seems to stabilize until P4, where it stands at 73%, which is not significantly different from the loss seen at E14.5.

These results suggest that the geniculate ganglion loss is susceptible to neurotrophin signalling during the initial stages of gustatory development, as the two most important factors, BDNF and NT4 exert their influence by E14.5 and then

cause no further loss. Similarly, the loss of geniculate ganglion neurons in animals with a point mutation in TrkB/Shc docking site causes a loss of neurons initially at E12.5, which increases by E14.5 upon which it is stabilized. This is also consistent with experiments done previously showing the higherst amount of cells death at E14.5 during normal mouse development (Carr et al. 2005).

4.1.5 Other systems dependable on signalling via TrkB/Shc docking site

Previous experiments showed that the involvement of the TrkB/Shc docking site does not have the same extent in every ganglion. Examination of the vestibular ganglion found no loss of neurons at P0 (when the loss of neurons in *Trkb* null mutants was nearly 60% at the same stage), suggesting that survival signalling occurs through a docking site different from TrkB/Shc, although some involvement was shown in maintenance of these neurons as a proportion was lost after birth at P7 (Minichiello et al. 1998). The same study showed that the TrkB/Shc docking site mutation caused loss of nodose neurons at 60%, similarly to what has been shown in *Nt4* null and *Bdnf* null mice (see Introduction, Conover et al. 1995, Liu et al. 1995, Fritzsch et al. 1997). In this case the TrkB/Shc docking site was able to facilitate survival signalling of NT4-dependent neurons while BDNF-dependent neurons appeared unaffected. TrkB/Shc docking site influence in NT4-dependent neuronal survival was further shown by a loss of D-hair receptors, mechanoreceptors innervating vellus hairs, which are fully NT4-dependent. The loss of these receptors was identical to that seen in *Nt4* null mice (Minichiello 1998).

TrkB/Shc docking site is therefore involved in regulating neuronal survival in some peripheral nervous system ganglia such as the geniculate and nodose, but not in others, such as vestibular ganglion. Our experiments show that TrkB/Shc is not only able to facilitate survival signalling of NT4, as TrkB/Shc point mutation lost a number of geniculate ganglion neurons at the time of NT4 influence. It is also able to facilitate survival signalling of BDNF, as *Bdnf* null mice lose about half of their

geniculate ganglion neurons by E14.5. Furthermore, TrkB/Shc docking site is the only site involved in facilitating geniculate ganglion neuron survival.

4.1.6 The geniculate ganglion neuronal survival may be supported by other factors

In our and other's studies it was found that geniculate ganglion is crucially dependent on signalling through the TrkB receptor in terms of neuronal survival. However a complete loss of neurons was not found in any of the mutations involving TrkB receptor. We have shown that the *Trkb*$^{D/D}$ mice lose large proportion of their neurons starting early in development, at E12.5, with the loss continuing throughout development until the gustatory development is complete. We weren't able to examine older animals because the *Trkb*$^{D/D}$ animals are not viable and die shortly after birth. In these and previous experiments, animals with mutations in *Trkb*, *Bdnf* and *Nt4*, and both docking sites, have been shown to lose a large majority of neurons, up to 90% (Liu et al. 1995, Conover et al. 1995, Erickson et al. 1996, Fritzsch et al. 1997), but the neuronal population was never completely lost.

There are several reasons why the geniculate ganglion neurons may not be completely lost in any of the mutant animals discussed previously. One is the involvement of p75NTR. P75NTR is a high-affinity receptor for pro-forms of neurotrophins, and a low-affinity receptor for mature neurotrophins. It has been shown to be co-expressed with Trk receptors in many neuronal populations (See Introduction, Bibel et al. 1999, Bibel and Barde 2000), and it's signalling has been often associated with apoptosis, mostly with pro-forms of neurotrophins (see Introduction, among others Nykjaer et al. 2004, Kenchappa et al. 2006). It has been shown that animals that lack this receptor, *p75* null mice, lost 25% of geniculate ganglion neurons by postnatal day 7 (P7) (Krimm 2006). This suggests that some of the neurons present in the geniculate ganglion may be dependent on signalling via the p75NTR receptor. Whether this receptor is important for the survival of the geniculate ganglion neurons during development, or whether the remaining

neurons in *Trkb* null, *Trkb*$^{D/D}$ mice and *Bdnf/Nt4* null mice are p75NTR-dependent, or at least supported by p75NTR signalling, remains to be seen.

Another possibility is the influence of a different neurotrophin in the geniculate ganglion. NT3 has been previously found to influence survival of these neurons, as *Nt3* null mice have been shown to lose about 25% (Farinas et al. 1994) to 45% of geniculate ganglion (Liebl et al. 1997) by P0, and this loss was exacerbated in animals with double mutation of *Bdnf/Nt3*, where the loss of geniculate ganglion neurons rose to 65% (Liebl et al. 1997) (a more severe loss, 100%, was shown in the vestibular ganglion in the same animals, Ernfors et al. 1995). This suggests that the geniculate ganglion contains a subpopulation of neurons that may be NT3-dependent. The role of the receptor for this ligand has also been postulated. TrkC has been shown to be expressed in the geniculate ganglion in rat (Cho and Farbman 1999). *TrkC* null newborn animals lost 11% of geniculate ganglion neurons. The loss of neurons in *Nt3* null mice therefore exceeds the loss of neurons in mice with a null mutation in NT3's primary receptor, TrkC, suggesting that NT3 is able to support some TrkB-expressing neurons. This is also supported by data from culture experiments where it was shown that 15% of neurons required NT3 for survival (compared to 80% requiring BDNF or NT4), (Al-Hadlaq et al. 2003). However, the dependency of these neurons on NT3 during gustatory development were not examined and it is therefore not known whether NT3 causes a subpopulation of neurons to survive in the heavily-affected *Trkb* null, *Trkb*$^{D/D}$ and *Bdnf/Nt4* double null mutant animals.

4.2 Innervation into the tongue

Assessment of target innervation was done at three different developmental time points in order to encompass all stages of gustatory development. The first stage, E16.5, was examined to analyze the influence of point mutations in the TrkB receptor docking sites on target innervation after the initial period of innervation at E14.5. Second stage, newly born animals, would highlight any deficits present in these animals at a point when the innervation to the tongue is complete, although the development of taste buds is not finalized and some are still undergoing morphological changes (Sun and Oakley 2002, Shuler et al. 2004). Finally, examination of target innervation in adult animals would highlight any prolonged influence that a point mutation in either the TrkB/Shc or TrkB/PLCγ docking site would have on a fully developed mouse gustatory system.

Innervation into the taste buds is not homogeneous and depends on the fungiform papillae location

In order to analyze the amount of innervation into the neural and taste buds we decided to evaluate the amount of innervated neural buds (at E16.5) and taste buds (at P0 and adult stages) present on the whole tongue. To understand the innervation patterns, due to the fact that the geniculate ganglion afferents innervate the anterior-most two-thirds of the whole tongue, we decided to split the tongue into two areas, the tip of the tongue and the middle of the tongue (see Results, Figure 6), so that the whole area of fungiform papillae localization on the tongue would be encompassed. The importance of this division was shown in previous studies, for example Mistretta et al. 1999 showed that tips of tongues in *Bdnf* null mice lost proportionately less taste buds than intermediate parts of the tongue. Guagliardo et al. 2007 also showed that taste buds present in the tip of the tongue are more sensitive to chorda tympani sectioning. However, many studies focusing on innervation into the tongue did not evaluate the difference between

the two areas despite showing differences in innervation between genotypes (e.g. Lopez et al. 2006, Ma et al. 2009 and others) and the precise relationship between the innervating patterns and the localization of taste buds on the tongue is not known.

Tracing the geniculate ganglion afferents into the tongue

In order to proceed with this study we needed to find a reliable method that would enable us to quantify the amount of innervation into the tongue and the amount of innervated taste buds present on the tongue. Initial stages of these experiments were done by anterograde tracing of lipophilic carbocyanine DiI. In this method, the geniculate ganglion is labelled with crystals of this tracer, which is then transported via the geniculate axons into the tongue (e.g. Ma et al. 2009, Patel et al. 2010 and others). The DiI crystals are placed directly above the geniculate ganglion of animals of desired age, and after the transport into the tongue the innervation is visualised and quantified (see Methods). This method, however, proved unsuitable for these experiments due to several different reasons. On more than a few occasions the DiI tracing resulted in an incomplete labelling of the tongue, likely due to a defect in dye transport into the tongue. This, in turn, could have been caused by incomplete labelling of the geniculate ganglion which caused only a proportion of the afferents to be labelled by this dye. Often we observed large background labelling of the entire tongue. This could have been due to prolonged incubation time required for the transport of the dye into the tongue as the timing of the incubation is very tentative and requires hit-or-miss methodology as the incubation times can be very long (up to several weeks depending on the stage of the embryo, see Methods). However, it could also results from labelling of the trigeminal ganglion which provides somatosensory innervation into the tongue epithelia and is located directly adjacent to the geniculate ganglion. This led to large inconsistencies even among animals of the same genotypes. In order to avoid this problem we tried different applications of the dye to label the ganglion, some of which included

dissection of the brain or inserting the dye caudally to the facial nerve, excluding brain dissection. Due to large variability between experiments and unreliability of a consistent labelling of the tongues this method was abandoned.

We therefore decided to use a different approach to evaluate the amount of innervated taste buds present on the tongue. Several studies used immunohistochemistry to visualize the presence of taste buds on tongue sections (e.g. Ito et al. 2010, Nosrat et al. 2012 and others). In our experiments we have chosen immunofluorescence method and used antibodies to detect innervation as well as presence of neural and taste buds (see Methods). This method proved to be both sufficient and reliable for the analysis of innervated taste buds at all stages analysed.

4.2.1 Innervation into the tongue at E16.5

Point mutation in TrkB/PLCγ docking site causes deficits in innervation of the tip of the tongue but not in the middle

Examining the innervation at E16.5 we found that animals with a point mutation in TrkB/PLCγ docking site showed a deficit in the amount of innervated neural buds present on their tongues. Interestingly, this did not apply to all of the regions of the tongues. The most affected region of the tongue was the tip. While examination of the middle part of the tongue revealed no difference between TrkB/PLCγ docking site mutant embryos compared to the wild type control embryos. This suggests that the tip of the tongue was more affected by the loss of signalling downstream of the TrkB/PLCγ docking site.

Previous work by Mistretta et al. 1999 showed that the number of taste buds present in mice with a *Bdnf* null mutation was lower when compared to the wild type animals; however the deficits in taste bud numbers were not as severe in the tip of the tongue when compared to the intermediate regions of the tongue in these animals. Interestingly, Guagliardo et al. 2007 showed innervation deficits in mice after chorda tympani transsection, where the taste buds present on the tip of

the tongue were more severely affected than those present in the middle part of the tongue. Therefore, some aspects of the regional differences of the taste buds present on the tongue may be explained by either neurotrophin support or the amount of afferents able to innervate these taste buds. However, all the studies mentioned here examined only postnatal mice and did not take into account the embryonic development of the gustatory system so targeting into the tongue could not be taken into account.

BDNF may regulate target innervation via TrkB/PLCγ docking site

One of the possibilities that explain the deficits in targeting is the lack of BDNF signalling. BDNF was found to influence the targeting into the tongue during embryonic development, while NT4 was found not to have such an influence as *Bdnf* null mutant mice showed deficits in targeting starting at E14.5. At this point, the branching of the geniculate afferents was increased (discussed below, Ma et al. 2009). These deficits in innervation were seen at a stage where already half of the geniculate ganglion neurons were lost in these animals (Patel et al. 2010). In animals harbouring a point mutation in Trkb/PLCγ docking site the signalling pathways of BDNF regulating targeting may be disrupted. Since BDNF is important during the guidance of geniculate ganglion afferents into the tongue, it is possible that the fibers from the geniculate ganglion are able to innervate the tongue, however insufficient neurotrophin support due to the lack of the TrkB/PLCγ docking site and thus signalling facilitated by it, causes larger deficits in targeting in the tip of the tongue compared to the middle. These results suggest that due to a lack of deficits in geniculate ganglion neuron survival in animals with a point mutation in the TrkB/PLCγ, the targeting deficits reflect altered fiber guidance rather than a lower amount of available geniculate afferents.

Examining animals with a double docking site mutation, $Trkb^{D/D}$, we found large deficits in the amount of innervated neural buds. This was true for all the tongue regions analysed: tip, middle and whole tongue. Interestingly, the amount of

innervated neural buds was not significantly different between *Trkb*$^{D/D}$ mice and *Trkb*$^{P/P}$ mice when looking at the tip area of the tongue. This suggests that it is the TrkB/PLCγ site that determines the amount of innervation into the tip of the tongue. There are, however, other factors that play a role in this process.

First, *Trkb*$^{D/D}$ mice showed large deficits in geniculate ganglion neuron survival at ages even prior to innervation, at E12.5 and E14.5. It therefore cannot be determined from this data whether the losses are accounted for by the loss of geniculate ganglion neurons only, or by the loss of neurons and deficits in targeting.

Second, mice with a point mutation in the TrkB/Shc docking site were not examined at this developmental stage. Considering the substantial loss of geniculate ganglion neurons seen at both E12.5 and E14.5, we would expect a lower amount of neural buds present on these tongues as well. This suggests that the amount of innervated neural buds would show deficits in numbers as well.

Interestingly, the TrkB/PLCγ docking site has been found to influence innervation in different systems as well. Experiments done by Sciaretta et al. 2010 showed that in the vestibular system, animals with at point mutation in this docking site showed aberration in fiber trajectories into the vestibular sensory epithelia at P0 and a week old mice. In these *Trkb*$^{P/P}$ mice the fibers would extend along the perimeter and circle around the sensory epithelia during innervation into the calyx of the inner ear, suggesting a disorientation of afferent fibers in mice with a point mutation in the TrkB/PLCγ docking site. These experiments therefore highlighted the importance of TrkB/PLCγ docking site in the guidance of sensory afferents into their targets, such as the geniculate ganglion afferents into the tongue.

Representation of neural buds is affected in *Trkb*$^{D/D}$ mice, but not in *Trkb*$^{P/P}$ mice

The localization of taste buds in mice is conserved among individuals: the taste buds on the tongue are arranged in rows and columns (Jung et al. 2004, Zaidi and Whitehead 2006). This localization results in a specific “map” of taste buds

present on the tongue, creating a conserved number of taste buds present on the tip of the tongue as well as the middle of the tongue. Our experiments examined the possibility of point mutations in TrkB receptor docking sites influencing the positioning of the neural and taste buds on the tongue. We approached the evaluation as a representation of innervation neural buds that were located on the tip of the tongue compared to the total amount of innervated neural buds present on the whole tongue. We found that at a stage just after the initial innervation of geniculate ganglion fibers into the taste buds, the point mutation in the TrkB/PLCγ docking site did not influence the amount of innervated neural buds represented in the tip of the tongue. Examining animals with a point mutation in both docking sites we found that the innervation of the tip of the tongue was exaggerated compared to both the wild type animals as well as animals with a point mutation in the TrkB/PLCγ docking site.

These results suggest that while TrkB/PLCγ site influences the targeting of the neural buds on the tongue, it is not involved in determining the localization of the neural buds at this stage of development as the proportional presence of neural buds in the two areas of the tongue is not altered despite the innervation deficits seen in these embryos. Because animals with a mutation in both the TrkB/PLCγ and the TrkB/Shc docking sites did show a difference in proportion of neural buds represented in the tip of the tongue, it is likely that the TrkB/Shc docking site is involved in signalling that determines the topographical map of neural buds on the tongue. As mentioned previously, experiments using *Bdnf* null mutant mice showed that this proportional presence is altered in adult animals (Mistretta 1999), and *Bdnf* null mutant animals showed similar deficits in innervation at this embryonic stage (Ma et al. 2009). Even though the influence of this mutation on the neural bud localization was not examined at this developmental stage, it can be hypothesized that BDNF signalling via the TrkB/Shc docking site influences the proportional presence of neural buds on the tongue at this stage. However, in order to prove this hypothesis, proportional innervation in *Bdnf* null mutants in embryonic stages needs to be examined.

4.2.2 Innervation into the tongue at P0

Animals with a point mutation in TrkB/PLCγ docking site show rescue in taste bud innervation at P0

Examination of innervated taste buds at P0 revealed striking differences compared to an earlier stage. Animals with a point mutation in TrkB/PLCγ docking site showed a significantly higher amount of innervated taste buds present on their tongues when compared to the wild type animals. This was also true in each of the different areas of the tongue; the tip and the middle. Therefore, the innervation into the taste buds in mice with a point mutation in TrkB/PLCγ docking site was rescued between E16.5 and P0, and increased when compared to the control animals.

Similar situation in terms of recovery of innervation was found in *bdnf* null animals before (Ma et al. 2009). At E14.5 a deficit in targeting into the neural buds was observed, with very few neural buds receiving the innervating fibers. At this stage, large amount of branching was observed in the epithelial regions of the tongue. This was seen to increase at E16.5 and a small number of innervated fungiform papillae was innervated, however, the total amount of innervated neural buds was still significantly lower when compared to the wild type animals. By E18.5 more fungiform papillae were innervated, while the excessive branching in the epithelium was decreased. This suggests that BDNF crucial for target innervation of developing mice.

Experiments by Ma et al. (2009) also showed that NT4 itself was not involved in targeting into the taste and neural buds on the tongue as the numbers of innervated buds remained unchanged in *nt4* null mice. This is interesting, because *nt4* null animals lost 50% of the geniculate ganglion neurons (Lopez et al. 2010), while the target innervation appeared to be normal (Ma et al. 2009), suggesting that despite lower amounts of available fibers innervating the tongue are still able to innervate normal amount of taste buds in a sufficient manner in

order for them to be maintained. NT4 was therefore rather involved in branching of these afferents in the tongue as animals with a double *bdnf/nt4* null mutation have lost both targeting and branching in the tongue. Although the amount of target innervation lost was higher in the double mutant when compared to *bdnf* null mutants, suggesting that NT4 can influence targeting only when the mice already lack BDNF.

While experiments with *bdnf* null mutants did show a certain amount of recovery of neural bud innervation between E16.5 and E18.5, Ma et al. (2009) concluded that this was due to the large increase in chorda tympani branching just below the tongue epithelium causing some fibers to penetrate this epithelium and innervate some of the neural buds by chance. The amount of neurons surviving in the geniculate ganglia of *bdnf* null mice was much lower, as was the amount of innervation rescue when compared to what was observed in $Trkb^{P/P}$ mice at P0. While this is a later developmental stage, it is unlikely that a large amount of innervation rescue would be observed in *bdnf* null mice in such a short amount of time. This suggests that this docking site may be involved in timing of the innervation rather than determining the outcome of innervation development. We were unable to quantify the amount of innervation present in the tongue in $Trkb^{P/P}$ mice, which would help in identifying the precise role of TrkB/PLCγ docking site in targeting.

Animals with a point mutation in TrkB/Shc and both TrkB/PLCγ and TrkB/Shc docking sites show deficits in innervated taste buds numbers

Examining animals with a point mutation in TrkB/Shc docking site showed large deficits in number of innervated taste buds present on the tongue in all the previously defined regions. Since we found extensive losses in the geniculate ganglion neuron survival in these mice at all stages examined prior to, during and after the onset of innervation, we concluded that the deficits seen in these mice

were due to the lack of fibers from the geniculate ganglion rather than a deficit in targeting.

Similar situation was shown in *Trkb*$^{D/D}$ mice, which showed large deficits in both innervation and survival of the neurons in the geniculate ganglion. Comparisons of innervated taste buds between the *Trkb*$^{S/S}$ and *Trkb*$^{D/D}$ mice revealed no significant difference in the amount of innervated taste buds present in the whole tongue (56.3±3.5 vs. 38.7±7.57 respectively, n=3 in both cases, p=0.021), and the middle (4±1 vs. 9.3±7.6, p=0.293), but were significantly different from each other in the tip where animals with a TrkB/Shc docking site point mutation showed higher amount of taste buds (52.3±3.2 vs. 29.3±7.6, p <0.01). Due to the genetic strategy used to generate these mutants we cannot compare them directly, which may be the reason we observe the disparity between the innervated taste bud numbers seen in the tip of the tongue in these animals. However, these results suggest that the Trkb/Shc adaptor site is important for regulation of the geniculate ganglion neuron survival and a point mutation causes, possibly consequentially, deficits in neuronal innervation as well. Whether this is due to the lack of available fibers for target innervation or due to the fact TrkB/Shc docking site is also involved in regulating the target innervation is not known. To determine the influence of this site on target innervation, branching patterns in animals lacking TrkB/Shc docking site need to be examined.

TrkB/Shc facilitates differential survival of taste buds on the tip of the tongue but not in the middle

As with previous developmental stage we examined the possibility that mutations in the docking sites altered the topographical maps of innervated taste buds present on the tongues. The analysis revealed no difference in innervated taste buds representation in the tip of the tongue in animals with a point mutation in the TrkB/PLCγ docking site or both docking sites when compared to the wild type control animals, suggesting that the TrkB/PLCγ docking site is not involved in

influencing the distribution of the innervated taste buds on the tongue. While we found no difference between the wild type control mice and mice with double docking site mutation, we did find a significant difference in animals with a point mutation in the TrkB/Shc docking site: these animals had a higher proportion of innervated taste buds in the tips of the tongues compared to wild type animals. This suggests that the point mutation in the TrkB/Shc docking site not only causes a loss of total number of innervated taste buds at this stage together with a lack of surviving geniculate ganglion neurons at all stages throughout the gustatory development, but it also causes a disruption of the conserved distribution of taste buds on the tongue. Previous experiments (as mentioned above) showed that BDNF affects the representation in the taste buds in the pre-defined regions of the tongue in adult animals (Mistretta et al. 1999). We hypothesized that because *Trkb*$^{D/D}$ animals were affected at E16.5 but *Trkb*$^{P/P}$ animals were not, the TrkB/Shc docking site played an important role in determining the localization of taste buds. The experimental data shown here supports this notion.

Why it is only the *Trkb*$^{S/S}$ animals that have an altered proportion of innervated taste buds on the tongue is not known. It is possible that in animals that lack a functional TrkB/Shc docking site, there is a deficient innervation into the tongue due to the lack of sufficient amount of geniculate ganglion neurons. In this case the TrkB/PLCγ docking site may be substituting the TrkB/Shc docking site in branching, leading to an increased amount of innervated taste buds in the tip of the tongue, as we showed that TrkB/PLCγ site has a marked influence on the tip of the tongue rather than the middle of the tongue. Since the *TrkB*$^{D/D}$ animals do not possess a functional TrkB/PLCγ site, this recovery is not observed.

Another possibility is the influence of the TrkB/Shc docking site itself. BDNF was found to influence taste buds to a variable degree depending on their location; the tip of the tongue contains higher amount of taste buds proportionately to the intermediate regions in adult animals lacking BDNF (Mistretta 1999). We found a very similar effect in animals with a point mutation in the TrkB/Shc docking site in our experiments, albeit in an earlier developmental stage. This suggests that BDNF

signals via TrkB/Shc docking site to determine the survival of taste buds in the tip of the tongue to a higher degree than taste buds in the middle of the tongue. To confirm this hypothesis an examination of adult animals would be performed.

Point mutations in TrkB receptor docking sites do not influence the proportion of innervated taste buds on the tongue at P0

It has been shown before that during development fibers from the gustatory ganglion innervate the tongue and then target the previously formed neural buds (Mbiene et al. 1997, Ma et al. 2009, Ito et al. 2010). During this process the innervation of neural buds is not homogeneous, and throughout gustatory development the branching of these axons changes as they are stabilized and pruned. This causes some of the neural and eventually taste buds to not be innervated causing them to degenerate and to be then lost shortly after birth (Lopez and Krimm 2006, Zaidi and Whitehead 2006).

We set out to determine whether point mutations in the TrkB receptor docking sites TrkB/PLCγ and TrkB/Shc influence this process. To this end we examined the total number of uninnervated taste buds present in all the regions in all the genotype. We found no difference in the amount of uninnervated taste buds present on the tongues of mice with a point mutation in the TrkB/PLCγ docking site, or with a double docking site mutation when compared to the wild type control mice. This suggests that while TrkB/PLCγ site is heavily involved in the innervation and targeting process into the tongue throughout development, it does not alter the amount of taste buds that remain uninnervated on the tongue.

We could speculate whether there are taste buds present on the tongue that are determined to be uninnervated as it appears that even with a higher amount of innervation into the tongue as seen in animals with a point mutation in the TrkB/PLCγ docking site, the number of uninnervated taste buds stays constant. Similar effect is seen in *Trkb*$^{D/D}$ animals. Even though the amount of innervated taste buds is much lower than in any other genotype, the proportion of

uninnervated taste buds does not change, although a large variability was observed when accounting for the uninnervated taste buds.

Mice with a point mutation in the TrkB/Shc docking site showed lower amount of uninnervated taste buds when compared to the wild type animals. The variability of uninnervated taste buds was much lower than in the previously examined animals. This suggests that the point mutation in the TrkB/Shc docking site may be causing previously uninnervated taste buds to become innervated despite the overall low amount of innervated taste buds on the tongue. However since the total amount of taste buds on the tongues of all these animals was very low, we wanted to see whether the amount of uninnnervated was proportionately different in any of the mutant animals.

To show whether the number of uninnervated taste buds was in fact different among the genotypes we examined the proportion of innervated taste buds to the total amount of taste buds in all genotypes on the whole tongue. We found that the proportion of uninnervated taste buds was not different in any of the animals examined. These results suggest that each tongue contains a certain amount of taste buds that remain uninnervated until a certain point of development, even when the amount of fibers extending into the tongue is very low as shown in $Trkb^{S/S}$ and $Trkb^{D/D}$ animals. This is interesting because during development, some axons extending from the geniculate ganglion have been shown to innervate incorrect areas of the tongue, such that certain parts of the tongue contain too many innervating fibers while others lack innervation altogether, suggesting a period of rearrangement after the initial stages of innervation (Lopez and Krimm 2006). Our results show that the mechanism guiding the geniculate fibers into the tongue, while independent of signalling through either TrkB/Shc or TrkB/PLCγ docking site, is dependent on the amount of fibers available to innervate the taste buds as the proportion of uninnervated taste buds is conserved in all genotypes. Which factors play a role in this process remains to be determined.

Our results are consistent with previous experiments showing that by birth there is only a very low amount of uninnervated taste buds present on the tongue,

despite a degree of disparity between these experiments. Previously, Patel and Krimm (2006) found that by E18.5 there are only 2% of uninnervated taste buds present on the tongue in wild type animals while our results shown that by P0 this number is closer to 10%. However, the studies agree that by birth the vast majority of taste buds are innervated. Our experiments expand this further by showing that the amount of uninnervated taste buds is proportionally dependent on the total amount of taste buds, and even disruptions in innervation patterns do not curb this process.

4.2.3 Innervation into the tongue of adult animals

TrkB/PLCγ docking site may be involved in timing of innervation during development

We examined taste bud innervation in adult animals of all genotypes to determine whether the point mutations in the TrkB/Shc and TrkB/PLCγ docking sites have an influence on the gustatory system after the development has been completed. Interestingly, we found that the amount of innervated taste buds in mice with a point mutation in TrkB/PLCγ docking site was not different from the wild type control animals in any part of the tongue, while animals with a point mutation in TrkB/Shc docking site showed lower amount of innervated taste bud than wild type mice in all aspects of the tongue. Animals with a double mutation in TrkB/PLC and TrkB/Shc docking sites were not examined because these animals are not viable and do not survive to adulthood.

These results suggest that TrkB/PLCγ docking site is involved in timing of the innervation patterns in the tongue. This is shown by affected innervation at E16.5, in a period just after the innervation into the tongue. By birth this innervation is rescued where the amount of innervated taste buds is shown to be even higher than those of the control animals, and by adulthood the innervation is returned to normal state. This could mean a normal development of innervation in mouse

tongue in animals lacking a functional TrkB/PLCγ docking site, however, development that is delayed when compared to control animals. Whether the functionality of the rescued taste buds is affected remains to be seen (discussed below). The deficit and subsequent rescue by delayed innervation is quite different from what has been observed in the vestibular system where the point mutation in the TrkB/PLCγ docking site was shown to cause deficits in target innervation that lasted into adulthood, without any rescue being observed (Sciaretta et al. 2010).

TrkB/Shc docking site, on the other hand, influences the survival of the geniculate ganglion neurons at all stages of gustatory development, which leads to a lack of innervated taste buds. Because we do not see any recovery from the neuronal death during the development or in adulthood, the deficits in innervation persist into adulthood as well.

TrkB/Shc docking site influences the proportional representation of taste buds in different regions of the tongue in adult mice

Similarly to previous developmental stages we examined the proportion of taste buds in either the tip or the middle compared to the total amount of taste buds on the tongue of adult mice. We found that while taste buds in mice with a point mutation in TrkB/PLCγ docking site contained the same proportion of taste buds in the tip of the tongue as did the wild type control mice, this number was higher in mice with a point mutation in the TrkB/Shc docking site when compared to wild type animals. These results suggest that TrkB/PLCγ site does not influence the innervation into the taste buds differently in various parts of the tongue, while the geniculate ganglion neuron loss seen in animals lacking functional TrkB/Shc docking site causes a disproportionate loss of taste buds in the middle of the tongue compared to the wild type animals. Because experiments with embryonic animals focusing on proportional innervation into the tongue have not been done, we hypothesized that the TrkB/Shc docking site may be facilitating the role of BDNF as adult animals with a *Bdnf* null mutation retained a higher amount of taste buds in

the tip of the tongue (Mistretta et al. 1999). When comparing animals of the same stage, we found that *Trkb*$^{S/S}$ adult mice showed similar effect to that showed in *Bdnf* null adult animals. This led to conclusion that while during development *Trkb*$^{S/S}$ animals resemble the effects of *Bdnf* knockout, they last until adulthood to at least a certain degree. To show neurotrophin dependency, proportional analysis needs to be done in embryonic animals. Why the numbers of innervated taste buds in the tip of the tongue are disproportionately lower is not clear. However, higher sensitivity of these taste buds to denervation was previously shown in adult animals by Guagliardo et al. 2007. Since the amount of geniculate ganglion neurons in these animals is much lower compared to control animals, it could offer an explanation to why the amount of taste buds in the tip of the tongue is lower.

Virtually no uninnervated taste buds were found in any of the genotypes examined at this age. This suggests that by the time animals reach adulthood innervated taste buds are able to survive, while the uninnervated degenerate. These data are in agreement with previous findings suggesting that uninnervated taste buds are lost by adulthood (Nagato et al. 1995). Point mutations in either the TrkB/PLCγ docking site or the TrkB/Shc docking site do not appear to influence this process.

4.3 Taste bud morphology

4.3.1 Taste bud morphology in newly born animals

Innervating geniculate afferents determine the size of taste buds

Taste buds require at least two to seven innervating geniculate ganglion neuron afferents in order to survive, be maintained and function correctly (Zaidi and Whitehead 2006). Since we have seen very different phenotypes of innervation in our experiments we wanted to determine whether the differences in innervation would have a role on the morphology of taste buds.

Shape and size of a taste bud is influenced by the amount of neuronal fibers innervating it; higher number of fibers innervating a single taste bud causes this taste bud to become larger in size. Moreover, by examining the size of a taste bud one can predict the number of gustatory fibers innervating it (Krimm and Hill 1998). Due to the methodology used in our experiments we were able to examine the morphology of taste buds at birth and in adulthood to account for any morphological, and hence innervational, differences amongst the genotypes.

The assay of taste bud size was done in two dimensions: the width and height of taste buds were measured (at their widest and highest points, respectively) according to the Troma-I antibody, which is specific for cytokeratin 8 expression. This marker has been previously shown to be specific for differentiated taste buds in mice (Toh et al. 1993, Oakley et al. 1998) and was therefore chosen to determine the sizes of the taste buds. Expression of Troma-I has been shown to be specific to clusters of cells near developing neural buds already at E13.5 (Ito et al. 2010), however, the specificity for taste buds was not reached until after birth. We were therefore able to examine the morphology of newly born and adult taste buds. Similar methodology for assessing the morphology of taste buds has been used in experiments before (e.g. Sun and Oakley 2002, Patel et al. 2010b, Nosrat et al. 2012).

Because previous experiments showed that morphological characteristics of taste buds on the tip of the tongue are affected to a larger extent than those in more caudal regions by neurotrophin deficiency (e.g. Mistretta 1999, Patel 2010b), and chorda tympani sectioning (Guagliardo et al. 2007), we decided to extend our analysis to determining whether the morphological response to the loss of signalling downstream of TrkB receptor docking sites would be affected in the two separate regions on the tongues, the tip and the middle, of both newly born and adult mice.

Measuring the taste buds in newly-born animals, at P0, we found that the taste buds present on the tongues of animals with a point mutation in the TrkB/PLCγ site were not morphologically significantly different from their wild type counterparts, despite the fact that these mice showed a higher number of innervated taste buds in their tongues. This was true in all regions of the tongue, including the tip of the tongue and the middle of the tongue. The taste buds in $Trkb^{S/S}$ mice were, on the other hand, significantly smaller compared to wild type counterparts in all aspects. The same effect was seen when looking at the sizes of taste buds of $Trkb^{D/D}$ mice.

Therefore, despite the fact that we observed a higher number of innervated taste buds in $Trkb^{P/P}$ mice, taste buds present on the tongues of these animals did not increase in size. There are two possible explanations for this effect. First, the innervation into individual taste buds in $Trkb^{P/P}$ mice was not altered, which is why the sizes of the taste buds remained the same as an increase in innervation into individual taste bud would cause them to increase in size (Krimm and Hill 1998). Second, the taste buds in these mice have already reached a maximum size of a taste bud (hypothesizing that there is such a level), and thus even increased innervation would have no effect on the size of these taste buds. We were not able to quantify individual innervations to determine which possibility is true.

Identical hypothesis can explain the smaller size of taste buds seen in $Trkb^{S/S}$ and $Trkb^{D/D}$ animals. Because the amount of surviving geniculate ganglion neurons observed in these animals was lower than those of control animals, only a fraction of geniculate afferents was able to enter the tongue. This caused only a limited

amount of innervation available for taste buds present on all regions of the tongue containing the fungiform papillae (causing fewer fibers available for each taste bud), which led to a decrease in taste bud size and the similarity between the taste bud sizes of both *Trkb*$^{S/S}$ and *Trkb*$^{D/D}$ mice. This supports the idea that the amount of innervating fibers is correlated to the size of the taste buds, where low amount of innervating fibers causes a decrease in taste bud size. However, in order to directly show this, a quantitative measurement of innervation into individual taste buds is required to substantiate this point.

4.3.2 Taste bud morphology in adult animals

Point mutations in TrkB receptor docking sites show various phenotypes of taste buds in adult mice

In order to evaluate differences in taste bud morphology after the development of the gustatory innervation, taste buds of adult animals were examined. Animals with a point mutation in the TrkB/PLCγ site showed significantly larger taste buds than their controls in terms of their height but not width, but this was only true in the tip of the tongue and not in the middle. Taste buds in *Trkb*$^{S/S}$ mice, on the other hand, were larger in height compared to wild type animal, but smaller in width. In the tip the taste buds showed difference only in height, where the *Trkb*$^{S/S}$ animals were still larger, but not in width where we found no differences between the mutant animals and their controls. Taste buds in *TrkB*$^{D/D}$ animals were not examined because these mice are not viable and don't survive to adulthood.

These experiments suggest that a mutation in the TrkB/Shc docking site influences the morphology of the taste buds early after birth. In adulthood, however, this influence is lost and altered taste bud morphology is recovered to some extent. This is the case despite the fact that there is no indication of recovery of geniculate ganglion neurons in these mice. Taste buds in these mice were shown to be larger in height than those of wild type animals, but smaller in width. It is

unclear why this is the case, but it is possible that the recovery we observed may be altering the morphology of these taste buds. One way to examine this possibility would be to assess the number of taste cells present in these taste buds to see whether the point mutation in the TrkB/Shc docking site may cause a reorganization of these taste cells.

The TrkB/PLCγ docking site, on the other hand, does not influence the morphology of taste buds early after birth despite the large increase in innervation into the tongue seen in animals with a mutation in this site. In adulthood the height of the taste buds increases compared to the wild type controls while the width remains the same. The increase in size of the taste buds was only observed in the tip of the tongue. We previously hypothesized that the mutation in the TrkB/PLCγ docking site causes a developmental delay in neural and taste bud innervation that causes a higher amount if innervation at birth, but is nevertheless returned to normal by adulthood. It is therefore possible, that the higher amount of innervation into the taste buds at birth is reflected by an increased size of these taste buds after birth. When the innervation is returned to normal and the taste bud numbers fall to not significant difference compared to wild type animals, the taste buds remain larger. This suggests that the taste buds are able to increase in size, but then do not decrease back down and perhaps require a higher amount of geniculate fibers to be maintained. This is quite likely given the high amount of innervated taste buds seen at birth in these animals. In order to prove this, the amount of innervation into each taste bud in adult *Trkb*$^{P/P}$ mice needs to be shown in a more quantitative way, as well as possible degeneration of taste buds in these mice between birth and adulthood.

The suggestion that BDNF and NT4 act via TrkB docking sites TrkB/PLCγ and TrkB/Shc to influence taste bud morphology is supported also by previous experiments. Nosrat et al. (2004) showed that *Bdnf* null mice at birth contain fungiform papillae and taste buds smaller than those seen in wild type animals of the same age. Similarly, one week old *Bdnf* null mice were shown to have taste buds smaller in diameter compared to wild type counterparts (Sun and Oakley 2002,

Patel et al. 2010). Finally, this effect was seen in *Bdnf* null mice of two and three weeks of age (Mistretta et al. 1999). This study also showed that not only were the taste buds in these mice smaller in diameter, but they were also disproportionately concentrated on the very tip of the tongue suggesting that the tip of the tongue is more susceptible to morphological changes, as we have seen in adult animals with a point mutation in the TrkB/PLCγ docking site.

NT4 has also been shown to play a role in taste bud size in mice. Liebl et al. (1999) and Patel et al. (2010) showed that despite a significantly lower amount of innervated taste buds and fungiform papillae present on the tongues of P0 *Nt4* null mice compared to the wild type animals, the morphology of these papillae and taste buds was unaffected. This is interesting, because only half of the geniculate ganglion neurons were shown to survive at this stage in *Nt4* null mice (Patel et al. 2012), although the targeting of geniculate fibers was shown to be unaffected in these mice during development until after birth (Ma et al. 2009). The reason why the size of taste buds in *Bdnf* null animals is affected to a larger degree than the sizes of taste buds in *Nt4* null mice is perhaps because the defects in innervation are more extensive in *Bdnf* null mice. Deficits in *Nt4* null mice taste buds are not seen until adulthood, where it has been shown the taste buds decrease in size as a result of decreased innervation suggesting a role of NT4 in maintenance of taste buds rather than their targeting (Patel et al. 2012).

Ectopic overexpression of BDNF and NT4 was shown to lead to reductions in taste bud and fungiform papillae number in adulthood despite normal development at birth together with a respective 93% and 140% increase in geniculate ganglion number. One of the reasons this may be the case is the inability of the fibers to penetrate the epithelium of the tongue and the fibers approaching inappropriate targets due to the abundance of neurotrophin expression. This ultimately leads to degeneration of taste buds on the tongues of transgenic mice, which is also more apparent in the taste buds present on the tip of the tongue rather than in caudal regions, suggesting that both of these neurotrophins play a role in maintenance of taste buds on the tongue together with regulating the survival of geniculate

ganglion neurons (Krimm et al. 2001). A different study showed that despite the degeneration of taste buds in later life, specific overexpression of BDNF in mouse taste buds led to an increase in size of these taste buds at birth. Taste buds in these mice were assessed for their height and width and the authors found that BDNF-overexpressing mice had wider and not higher taste buds as a result of an increased number of cells in these taste buds (Nosrat 2012), which corresponded to a higher amount of innervation into the taste buds.

Our results, compared to results obtained from previous experiments suggest that it is both BDNF and NT4 that influence targeting via the TrkB/PLCγ site. The developmental delay that we observe in these mice in terms of target innervation is very similar to what has been observed in *Bdnf* null mice (Ma et al. 2009), and we can therefore hypothesize that with the loss of BDNF signalling via TrkB/PLCγ there is at first a loss of target innervation at E16.5, which is then rescued by birth. Experiments with *Bdnf* null mice showed a certain amount of recovery in mice at E18.5 (Ma et al. 2009), but the extent of this is never close to what we found in $Trkb^{P/P}$ mice. The recovery seen in TrkB/PLCγ mutant mice at birth can be perhaps explained by a differential need of both TrkB/PLCγ and TrkB/Shc docking sites. As $Trkb^{S/S}$ mice lose large amount of geniculate ganglion we are not able to determine whether the targeting in these mice is also affected. We can hypothesize that mice without a functioning TrkB/PLCγ docking site contain extensively branching geniculate fibers in the tongues, as seen in *Bdnf* null mice at the same age (Ma et al. 2009). At this point, BDNF may act via the TrkB/Shc docking site to guide the fibers into the actual taste buds; we observe the large amount of taste buds on the tongue due to the exaggerated innervation of the tongue by these fibers. In adulthood, maintenance of taste buds and their innervation is facilitated by NT4. It is therefore likely that NT4 acts via TrkB/Shc to prune connections into the taste buds that are not needed, causing the taste bud number to fall and become normal.

It is apparent that there are other factors influencing the morphology of taste buds as well. The size of taste buds was found to be influenced by NT3. NT3 is normally involved in lingual somatosensory innervation and not innervation into the

taste buds, but experiments with a double *Bdnf/Nt3* null mutation showed that the size of the taste buds was smaller than what was observed in *Bdnf* null mice (Nosrat et al. 2004). *Nt3* null mice were not found to have any gustatory deficits (Nosrat et al. 1997), and it is therefore likely that NT3 plays only a supportive role.

It was also found that the p75NTR receptor is important for taste bud development, as Krimm (2006) showed that mice lacking this receptor lose 26% of taste buds by adulthood. The effect this receptor had on the size of the taste buds was not determined, however, this receptor is another factor that may play a role in effects seen in animals with a point mutation in either TrkB/PLCγ or TrkB/Shc docking sites.

4.4 Conclusion

This study investigated the role of TrkB receptor docking sites on several aspects of rodent gustatory system. To this end, lines of mice with point mutations in TrkB/Shc docking site (*Trkb*$^{S/S}$), TrkB/PLCγ docking site (*Trkb*$^{P/P}$) or both docking sites (*Trkb*$^{D/D}$) were used.

Signalling pathways facilitated by the TrkB/Shc docking site play an important role in regulating the survival of geniculate ganglion neurons, as *Trkb*$^{S/S}$ mice lost large amount of neuronal population of this ganglion starting at E12.5. The neurons continued to be lost through the gustatory development until the development was completed several days after the birth of the animal. Signalling pathways downstream of TrkB/PLCγ docking site only have a supporting role in regulating the geniculate ganglion neuron survival through the gustatory development as no neurons were lost in *Trkb*$^{P/P}$ mice throughout development, but the amount of neurons lost in *Trkb*$^{D/D}$ animals was larger than in either *Trkb*$^{S/S}$ or *Trkb*$^{P/P}$ animals. Therefore, the survival signalling downstream of TrkB receptor for geniculate ganglion neurons is mediated by TrkB/Shc docking site. During early development of the geniculate ganglion at E12.5, this docking site facilitates signalling of NT4 which determines the survival rate of neurons in this ganglion. Several days later at E14.5, as the geniculate ganglion afferents extend their processes into the tongue, TrkB/Shc docking site facilitates signalling of both BDNF and NT4, which determines survival of a large proportion of the geniculate ganglion neurons. Due to the amount of neurons lost in *Trkb*$^{S/S}$ mice we concluded that neurons in the geniculate ganglion are either BDNF-, and/or NT4-dependent, however, none of these possibilities are mutually exclusive. A role of TrkB/PLCγ docking site has been suggested as a support for either substitution of stabilizing of signalling complexes downstream of TrkB receptor. Identical neuronal dependency is retained till the end of the gustatory system development. The crucial role of TrkB receptor in survival of geniculate ganglion neurons was demonstrated in mice with double docking site mutation.

Point mutation in the TrkB/Shc docking site also caused low amount of innervated neural and taste buds at all stages of development, presumably due to a deficit in geniculate ganglion afferents rather than alterations in targeting. TrkB/PLCγ docking site, on the other hand, determined the temporal development of the neural and taste bud innervation. Animals with a point mutation in this docking site presented developmental delay that followed a pattern of initial retardation (E16.5), increased branching (birth), and return to normal state (adulthood). It is therefore likely that the initial stages of innervation, until the birth of the animal are facilitated by BDNF signalling via TrkB/PLCγ docking site. This dependency is changed by adulthood, where the maintenance of innervation into the taste buds is likely determined by NT4 signalling via the same site. BDNF signalling through the TrkB/Shc docking site, on the other hand influences the topographical representation of taste buds on the tongue of animals as animals with a double docking site mutation showed overrepresentation of taste buds in the tip of the tongue throughout gustatory development.

Lastly, it was determined that low amount of innervating geniculate afferent fibers into the tongue in $Trkb^{S/S}$ and $Trkb^{D/D}$ (due to a low amount of surviving geniculate ganglion neurons) caused a decrease of taste bud sizes at birth. The taste bud size was partially rescued in $Trkb^{S/S}$ animals by adulthood ($Trkb^{D/D}$ animals are not viable). Geniculate ganglion neuron number was unaffected throughout development and adulthood in $Trkb^{P/P}$ animals, however, a developmental delay in innervation was observed, causing an increased number of innervated taste buds at birth. The size of taste buds did not change until adulthood, once the amount of innervated taste buds returned to normal. The taste bud size was increased in adult animals. While we weren't able to measure the innervation directly, we concluded that the large amount of innervating fibers causing an increased number of taste buds at birth caused, in turn, an increase in taste bud size in adulthood, once the taste bud number returned to normal.

Overall, our results suggest that the TrkB receptor has various roles in rodent gustatory development. While activated by its two ligands, BDNF and NT4,

the two docking sites present on this receptor, TrkB/Shc and TrkB/PLCγ play differential role in determining the survival of geniculate ganglion neuron population, amount of innervated neural and taste buds present on the tongues of these animals through development, as well as the actual morphology of these taste buds.

4.5 Future outlook

Future studies should determine the possible taste association of lost geniculate ganglion neurons. Recent experiments showed the geniculate ganglion containing spatially ordered gustotopic maps where each taste quality is encoded in its own stereotypical cortical field (Barretto 2012). Since we have seen large losses of geniculate ganglion neurons this approach would allow us to determine whether the neurons lost in either *Trkb*$^{S/S}$ or *Trkb*$^{D/D}$ are associated with specific tastes. Micro-endoscopy, together with two-photon imaging, could be used to shed light on the possibility that certain tastes are more important than others, i.e. the sweet taste important in suckling, and would therefore be less susceptible to loss of survival factors.

Branching analysis should be done on tongues of mice examined in these experiments. Namely, it is important to find out whether the phenotype in *Trkb*$^{P/P}$ mice during development mirrors the phenotype seen in previously–examined *Bdnf* null mice showing increased branching (Ma et al. 2009). *Trkb*$^{S/S}$ and *Trkb*$^{D/D}$ animals should also be assessed for branching quantity. We have concluded that the branching patterns are not affected in these mice and the neural and taste bud numbers are deficient due to low amount of geniculate ganglion neuron survival. It could be interesting to see whether the difference in functional TrkB/PLCγ docking site causes branching differences despite the fact that the innervation patterns are not different between the *Trkb*$^{S/S}$ and *Trkb*$^{D/D}$ animals. A difference in branching and therefore the amount of innervation might indicate a minimum amount required for sustaining a neural/taste bud population.

Quantitative analysis of innervation into individual taste buds will address the question whether it is in fact the amount of innervation into taste buds that determine their morphology. We concluded that since the amount of geniculate ganglion afferents innervating tongues is low in namely *Trkb*$^{S/S}$ and *Trkb*$^{D/D}$ animals, it is in fact lower amount of branching that causes the decrease of singular taste bud innervation and hence the size of these taste buds. We did not find any

difference in taste buds in *Trkb*$^{P/P}$ until adulthood. Because the height and width of taste buds were affected in the mutants used in the above experiments, the morphology and number of taste receptor cells should be examined using either electron microscopy or specific immunofluorescence. This will determine the extent of rescue seen in *Trkb*$^{S/S}$ animals, and the specific causes of changes in morphology seen in *Trkb*$^{P/P}$ animals from birth to adulthood.

Finally, examining the functionality of taste buds will determine whether the taste buds retained in *Trkb*$^{S/S}$ mice after birth are functional, and whether the loss of taste buds seen in these animals affects any specific tastes in particular. The same analysis performed on taste buds in *Trkb*$^{P/P}$ animals both at birth and in adulthood would reveal if the aberrant innervation into taste buds has caused changes in their functionality. Because P0 animals are too young for behavioural experiments, there are at least two possible strategies that can be used for this study. First, calcium imaging could be used to determine the responsiveness of taste receptor cells present on the taste buds of these animals to assess whether there are any differences between major tastant groups detection in the taste buds remaining on the tongue. Second possibility is to use micro-endoscopy in combination with two-photon imaging and mice with genetically engineered calcium sensors. Because the taste buds are able to trigger dedicated neural lines into and through the geniculate ganglion (Chen et al. 2011), this approach, as described by Barretto (2012), could be used to evaluate the representation of taste qualities directly in the geniculate ganglion. It could therefore be used to determine the functionality of the remaining taste buds as well as to determine whether specific classes of taste receptors cells are affected on the tongues of mice with point mutations in the TrkB receptor adaptor sites.

REFERENCES

Acheson A, Conover JC, Fandl JP, DeChiara TM, Russell M, Thadani A, Squinto SP, Yancopoulos GD, Lindsay RM (1995) A BDNF autocrine loop in adult sensory neurons prevents cell death. Nature 374: 450-453

Alcantara S, Frisen J, del Rio J a, Soriano E, Barbacid M, Silos-Santiago I (1997) TrkB signaling is required for postnatal survival of CNS neurons and protects hippocampal and motor neurons from axotomy-induced cell death. J Neurosci 17:3623–3633

Al-Hadlaq SM, Bradley RM, MacCallum DK, Mistretta CM (2003) Embryonic geniculate ganglion neurons in culture have neurotrophin-specific electrophysiological properties. Neuroscience 118: 145-159

Altman J, Bayer SA (1982) Development of the cranial nerve ganglia and related nuclei in the rat. Advances in Anatomy, Embryology and Cell Biology 74:1-90.

Arevalo JC, Conde B, Hempstead BL, Chao MV, Martin-Zanca D, Perez P (2000) TrkA immunoglobulin-like ligand binding domains inhibit spontaneous activation of the receptor. Molecular Cell Biology 20(16): 5908-16

Arevalo JC, Waite J, Rajagopal R, Beyna M, Chen ZY, Lee FS, Chao MV (2006) Cell survival through Trk neurotrophins receptors is differentially regulated by ubiquination. Neuron 50(4) 549-59

Arevalo JC, Wu SH (2006) Neurotrophin signalling: many exciting surprises! Cellular and Molecular Life Sciences 63(13): 1523-37

Bachmanov, A.A., Beauchamp, G.K., and Tordoff, M.G. (2002). Voluntary consumption of NaCl, KCl, CaCl2, and NH4Cl solutions by 28 mouse strains. Behavioural Genetics 32: 445–457.

Baker CV, Bronner-Fraser M (2001) Vertebrate cranial placodes I. Embryonic induction. Developmental Biology 232:1-61.

Balemans W, Van Hul W (2002) Extracellular regulation of BMP signalling in vertebrates: a cocktail of modulators. Developmental Biology 250:231-250

Balkowiec A, Katz DM (2002) Cellular mechanisms regulating activity-dependent release of native brain-derived neurotrophic factor from hippocampal neurons. Journal of Neuroscience 22: 10399-10407

Bamji SX, Majdan M, Pozniak CD, Belliveau DJ, Aloyz R, Kohn J, Causing CG, Miller FD (1998) The p75 neurotrophin receptor mediates neuronal apoptosis and is essential for naturally occurring sympathetic neuron death. Journal of Cell Biology 140:911–23

Barbacid M (1995) Structural and functional properties of the TRK family of neurotrophins receptors. Ann N Y Acad Sci 766:442–458.

Barretto R. (2012) Visualizing the neural representation of taste at the periphery: imaging ganglion function *in vivo*. XVI International Symposium on Olfaction and Taste. Stockholm, Sweden, 23.6.12-27.6.12

Bartel DL, Sullivan SL, Lavoie EG, Sévigny J, Finger TE (2006) Nucleoside triphosphate diphosphohydrolase-2 is the ecto-ATPase of type I cells in taste buds. Journal of Comparative Neurology 497:1–12

Bear MF, Connors BW, Paradiso MA (2007) Neuroscience: Exploring the Brain. Lippincott Williams & Wilkins, Third Edition

Beattie MS Harrington AW, Lee R, Kim JY, Boyce SL, Longo FM, Breshanan JC, Hemstead JC, Yoon SO (2002) ProNGF induces p75-mediated death of oligodendrocytes following spinal cord injury. Neuron **36**, 375–386

Begbie J, Brunet JF, Rubenstein JL, Graham A (1999) Induction of the epibranchial placodes. Development 126:895-902

Bibel M, Hoppe E, Barde YA (1999) Biochemical and functional interactions between the neurotrophin receptors trk and p75NTR. EMBO J 18:616–622.

Bibel M, Barde YA (2000) Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. Genes Dev 14:2919–2937.

Bitgood MJ, McMahon AP (1995) Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. Developmental Biology 172:126-138.

Boeshore KL, Luckey CN, Zigmond RE, LargeTH (1999) TrkB isoforms with distinct neurotrophins specificities are expressed in predominantly non-overlapping populations of avian dorsal root ganglion neurons. The Journal of Neuroscience 19:4739–47

Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME (1999) Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 96:857–868.

Carr VM, Sollars SI, Farbman AI (2005) Neuronal cell death and population dynamics in the developing rat geniculate ganglion. Neuroscience 134:1301-1308.

Chandrashekar J, Mueller KL, Hoon MA, Adler E, Feng L, Guo W, Zuker CS, Ryba NJ (2000) T2Rs function as bitter taste receptors. Cell 100:703–711

Chandrashekar J, Hoon MA, Ryba NJP, Zuker CS (2006) The receptors and cells for mammalian taste. Nature 44:288-294

Chandrashekar J, Yarmolisnky D, von Buchholtz L, Oka Y, Sly W, Ryba NJ, Zuker CS (2009) The taste of carbonation. Science 326(5951): 443-5

Chao MV (1994) The p75 neurotrophin receptor. The Journal of Neurobiology 25:1373–85

Chao MV, Hempstead BL (1995) p75 and Trk: a two-receptor system. Trends in Neuroscience 18 (7): 321-6

Chaudhari N, Roper SD (2010) The cell biology of taste. Journal of Cell Biology 190(3): 285-296

Cheal M, Oakley B (1977) Regeneration of fungiform taste buds: temporal and spatial characteristics. Journal of Comparative Neurology 172(4): 609-26

Chen X, Gabitto M, Peng Y, Ryba NJ, Zuker CS (2011) A gustotopic map of taste qualities in the mammalian brain. Science 33: 1262-6

Cho TT, Farbman AI (1999) Neurotrophin receptors in the geniculate ganglion. Molecular brain research 68: 1-13

Cohen S, Levi-Montalcini R, Hamburger V (1954) A nerve growth-stimulating factor isolated from sarcomas 37 and 180. Proceedings of the National Academy of Sciences 40 (10): 1014-1018

Conover JC, Erickson JT, Katz DM, Bianchi LM, Poueymirou WT, McClain J, Pan L Helgren M, Ip NY, Boland P, Friedman B, Wiegand S, Vejsada R, Kato AC, DeChiara TM, Yancopoulos GD (1995) Neuronal deficits, not involving motor neurons, in mice lacking BDNF and/or NT4. Nature 375: 235-238

Corbit KC, Foster DA, Rosner MR (1999) Protein kinase Cdelta mediates neurogenic but not mitogenic activation of mitogen-activated protein kinase in neuronal cells. Molecular Cell Biology 19:4209–4218

Corson SL, Hill DL (2011) Chorda tympani nerve terminal field maturation and maintenance is severely altered following changes to gustatory nerve input to the nucleus of the solitary tract. Journal of Neuroscience 31(21): 7591-603

Crowley C, Spencer SD, Nishimura MC, Chen KS, Pitts-Meek S, Armanini MP, Ling LH, McMahon SB, Shelton DL, Levinson AD (1994) Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell 76:1001–1011

Damak S, Rong M, Yasumatsu K, Kokrashvili Z, Varadarajan V, Zou S, Jiang P, Ninomiya Y, Margolskee RF (2003) Detection of sweet and umami taste in the absence of taste receptor T1r3. Science 301:850–853

Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91:231–241.

Datta SR, Katsov A, Hu L, Petros A, Fesik SW, Yaffe MB, Greenberg ME (2000) 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. Mol Cell 6:41–51.

Dawbarn D, Allen SJ (2003) Neurotrophins and neurodegeneration. Neuropathology and Applied Neurobiology 29(3):211-30

Dhouailly D, Xu C, Manabe M, Schermer A, Sun TT (1989) Expression of hair-related keratins in a soft epithelium: subpopulations of human and mouse dorsal tongue keratinocytes express keratin markers for hair-, skinand esophageal-types of differentiation. Experimental Cell Research 181(1): 141-158.

Doty RL (2012) Gustation. Wiley Interdisciplinary Reviews: Cognitive Science 3:29-46

Dvoryanchikov G, Tomchik SM, Chaudhari N (2007) Biogenic amine synthesis and uptake in rodent taste buds. Journal of Comparative Neurology 505:302–313

Erickson JT, Conover JC, Borday V, Champagnat J, Barbacid M, Yancopoulos G, Katz DM (1996) Mice lacking brain-derived neurotrophic factor exhibit visceral sensory neuron losses disctinct from mice lacking NT4 and display a severe developmental deficit in control of breathing. The Journal of Neuroscience 16(17): 5361-5371

Erickson RP (2000) The evolution of neural coding ideas in the chemical senses. Physiology and Behavior 69: 3–13 (2000).

Ernfors P, Lee KF, Jaenisch R (1994) Mice lacking brain-derived neurotrophic factor develop with sensory deficits. Nature 368:147-150

Esparis-Ogando A, Diaz-Rodrigues E, Montero JC, Yuste L, Crespo P, Pandiella A (2002) Erk5 participates in neuregulin signal transduction and is constitutively active in breast cancer cells overexpressing ErbB2. Molecular Cell Biology 22(1):170-85

Fan L, Girnius S, Oakley B (2004) Support of trigeminal sensory neurons by non-neuronal p75 neurotrophin receptors. Developmental Brain Research 150: 23–39

Farbman AI, Mbiene JP (1991) Early development and innervation of taste bud-bearing papillae on the rat tongue. Journal of Comparative Neurology 304:172-186

Farbman AI, Guagliardo N, Sollars SI, Hill DL (2004) Each sensory nerve arising from the geniculate ganglion expresses a unique fingerprint of neurotrophins and neurotrophins receptor genes. Journal of Neuroscience research 78:659-667

Farinas I, Jones KR, Backus C, Wang XY, Reichardt LF (1994) Severe sensory and sympathetic deficits in mice lacking neurotrophin-3. Nature 369:658–661.

Farinas I, Yoshida CK, Backus C, Reichardt LF (1996) Lack of neurotrohin-3 results in death of spinal sensory neurons and premature differentiation of their precursors. Neuron 17:1065-78

Figurov A, Pozzo-Miller LD, Olafsson P, Wang T, Lu B (1996) Regulation of synaptic responses to high-frequency stimulation and LTP by neurotrophins in the hippocampus. Nature 381 (6584): 706-9

Finkbeiner S, Tavazoie SF, Maloratsky a, Jacobs KM, Harris KM, Greenberg ME (1997) CREB: a major mediator of neuronal neurotrophin responses. Neuron 19:1031–1047

Fode C, Gradwohl G, Morin X, Dierich A, LeMeur M, Goridis C, Guillemot F (19980 The bHLH protein NEUROGENIN 2 is a determination factor for epibranchial placode-derived sensory neurons. Neuron 20:483-494.

Frade JM, Barde YA (1998) Nerve growth factor: two receptors, multiple functions BioEssays 20:137–45

Frade JM, Barde YA (1999) Genetic evidence for cell death mediated by nerve-growth factorand the neurotrophin receptor p75 in the developing mouse retina and spinal cord. Development 126:683–90

Fritzsch B, Sarai P, Barbacid M, Silos-Santiago I (1997) Mice with a targeted disruption of the neurotrophin receptor trkB lose their gustatory ganglion cells early but do develop taste buds. International Journal of Developmental Neuroscience 15:563-567

Ganchrow D, Ganchrow JR, Verdin-Alcazar M, Whitehead MC (2003) Brain-derived neurotrophic factor-, Neurotrophin-3-, and tyrosine kinase receptor=like immunoreactivity in lingual taste bud fields of mature hamster. Journal of Comparative Neurology 455: 11-24

Giger RJ, Wolfer DP, De Wit GM, Verhaagen J (1996) Anatomy of rat semaphoring III/collapsing-1 mRNA expression and relationship to developing nerve tracts during neuroembryogenesis. Journal of Comparative Neurology 375(3): 378-92

Goodman LJ, Valverde J, Lim F, Geschwind MD, Federoff HJ, Geller AI, Hefti F (1996) Regulated release and polarized localization of brain-derived neurotrophic factor in hippocampal neurons. Molecilar and Cellular Neurosciences 7(3):222-38

Gotz R, Koster R, Winkler C, Raulf F, Lottsperich F Schartl M, Thoenen H (1994) Neurotrophin-6 is a new member of the nerve growth factor family. Nature 372 (6503): 266-9

Griesbeck O, Canossa M, Campana G, Gartner A, Hoener MC, Nawa H, Kolbeck R, Thoenen H (1999) Are there differences between the secretion characteristics of NGF and BDNF? Implications for the modulatory role of neurotrophins in activity-dependent neuronal plasticity. Microscopy Research and Technique 45 (4-5): 262-75

Hall JM, Hooper JE, Finger TE (1999) Expression of sonic hedgehog, patched, and Gli1 in developing taste papillae of the mouse. Journal of Comparative Neurology 406:143-155.

Hall JM, Bell ML, Finger TE (2003) Disruption of sonic hedgehog signalling alters growth and patterning of lingual taste papillae. Developmental Biology 255:263-277

Hamanoue M, Middleton G, Wyatt S, Jaffray E, Hay RT, Davies AM (1999) p75-mediated NF-kappaB activation enhances the survival response of developing sensory neurons to nerve growth factor. Molecular and Cellular Neurosciences 14(1): 28-40

Harada S, Yamamoto T, Yamaguchi K, Kasahara Y (1997) Different characteristics of gustatory responses between the greater superficial petrosal and chorda tympani nerves in the rat. Chemical Senses 22(2): 133–140

Harada S, Yamaguchi K, Kanemaru N, Kasahara Y (2000) Maturation of taste buds on the soft palate of the postnatal rat. Physiology and Behavior 68(3): 333–339

He XL, Garcia KC (2004) Structure of nerve growth factor complexed with the shared neurotrophins receptor p75. Science 304 (5672): 870-5

Hellard D, Brosenitsch T, Fritzsch B, Katz DM (2004) Cranial sensory neuron development in the absence of brain-derived neurotrophic factor in BDNF/Bax double null mice. Developmental Biology 275:34-43

Hofer M, Barde YA (1988) Brain-derived neurotrophic factor prevents neuronal death in vivo. Nature 331 (6153): 261-2

Hofer M, Pagliusi SR, Hohn A, Leibrock J, Barde YA (1990) Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain. EMBO Journal 9(8): 2459-64

Holgado-Madruga M, Moscatello DK, Emlet DR, Dieterich R, Wong AJ (1997) Grb2- associated binder-1 mediates phosphatidylinositol 3-kinase activation and the promotion of cell survival by nerve growth factor. PNAS 94:12419–12424.

Hoshino N, Vatterott P, Eqwiekhor A, Rochlin MW (2010) Brain-derived neurotrophins factor attracts geniculate ganglion neuritis during embryonic targeting. Developmental Neuroscience 2(3): 184-96

Hosley MA, Hughes SE, Morton LL, Oakley B (1987) A sensitive period for the neural induction of taste buds. Journal of Neuroscience 7:2075-2080.

Huang ZJ, Kirkwood A, Pizzorusso T, Porciatti V, Morales B, Bear MF, Maffei L, Tonegawa S (1999) BDNF regulates the maturation of inhibition and the critical period of plasticity in mouse visual cortex. Cell 98:739–755

Huang EJ, Reichardt LF (2001) Neurotrophins: Role in neuronal development and function. Annual Review of Neuroscience 24: 677–736

Huang EJ, Reichardt LF (2003) Trk receptors: roles in neuronal signal transduction. Annual Review of Biochemistry 72: 609–642

Huang, YJ, Maruyama Y, Dvoryanchikov G, Pereira E, Chaudhari N, Roper SD (2007) The role of pannexin 1 hemichannels in ATP release and cell-cell communication in mouse taste buds. Proceeding of the National Academy of Sciences of the USA 104:6436–6441

Huang YA, Maruyama Y, Roper SD (2008a) Norepinephrine is coreleased with serotonin in mouse taste buds. Journal of Neuroscience. 28:13088–13093

Huang YA, Maruyama Y, Stimac R, Roper SD (2008b) Presynaptic (Type III) cells in mouse taste buds sense sour (acid) taste. Journal of Physiology 586:2903–2912

Huang YZ, Pan E, Xiong Z, McNamara JO (2008c) Zinc-mediated transactivation of TrkB potentiates the hippocampal mossy fiber-CA3 pyramid synapse. Neuron 57:546-558

Huang YA, Dando R, Roper SD (2009) Autocrine and paracrine roles for ATP and serotonin in mouse taste buds. J. Neurosci. 29:13909–13918

Huang T, Krimm RF (2010) Developmental expression of *Bdnf, Ntf4/5*, and *TrkB* in the mouse peripheral taste system. Developmental Dynamics 239:2637-2646

Huang YA, Grant J, Roper SD (2012) Glutamate may be an efferent transmitter that elicits inhibition in mouse taste buds. PLOS one 7(1): e30662

Ishida Y, Ugawa S, Ueda T, Yamada T, Shibata Y, Hondoh A, Inoue K, Yu Y, Shimada S (2009) $P2X_2$- and $P2X_3$-positive fibers in fungiform papillae originate from the chorda tympani but not the trigeminal nerve in rats and mice. The Jounal of Comparative Neurology 513:131-144

Ishimaru Y, Inada H, Kubota M, Zhuang H, Tominaga M, Matsunami H (2006) transient receptor potential family members PKD1L3 and PKD2L1 form a candidate sour taste receptor. Proceedings of the National Academy of Sciences of the USA 103(33): 12569-74

Iwatsuki K, Liu HX, Gronder A, Singer MA, Lane TF, Grosschedl R, Mistretta CM, Margolskee RF (2007) Wnt signaling interacts with Shh to regulate taste papilla development. Proceedings of the National Academy of Sciences of the USA 104:2253-2258.

Ito A, Nosrat IV, Nosrat CA (2010) Taste cell formation does not require gustatory and somatosensory innervation. Neuroscience Letters 471: 189-194

Ji Y, Lu Y, Yang F, Shen W, Tang TT, Feng L, Duan S, Lu B (2010) Acute and gradual increases in BDNF concentration elicit distinct signalling and functions in neurons. Nature Neuroscience 13 (3): 302-310

Johnson D, Lanahan A, Buck CR, Sehgal A, Morgan C, Mercer E, Bothwell M, Chao M (1986) Expression and structure of the human NGF receptor. Cell 47:545–554.

Jones KR, Farinas I, Backus C, Reichardt LF (1994) Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. Cell 76: 989-999

Jung HS, Oropeza V, Thesleff I (1999) Shh, Bmp-2, Bmp-4 and Fgf-8 areassociated with initiation and patterning of mouse tongue papillae. Mechanisms of Development 81:179-182

Jung H, Akita K, Kim J (2004) Spacing patterns on tongue surface-gustatory papilla. International Journal of Developmental Biology 48:157-161

Kandel E.R. (2001) The molecular biology of memory storage: a dialogue between genes and synapses. Science 294 (5544): 1030-8

Kao S, Jaiswal RK, Kolch W, Landreth GE (2001) Identification of the mechanisms regulating the differential activation of the mapk cascade by epidermal growth factor and nerve growth factor in PC12 cells. Journal of Biological Chemistry 276(21):18169-77

Kavanaugh WM, Williams LT (1994) An alternative to SH2 domains for binding tyrosine-phosphorylate proteins. Science 255(5192):1862-5

Kenchappa RS, Zampieri N, Chao MV, Barker PA, Teng HK, Hempstead BL, Carter BD (2006) Ligand-dependent cleavage of the P75 neurotrophin receptor is necessary for NRIF nuclear translocation and apoptosis in sympathetic neurons. Neuron 50:219– 232.

Kimball SR, Farrell PA, Jefferson LS (2002) Role of insulin in translational control of protein synthesis in skeletal muscle by amino acids or exercise. Journal of Applied Physiology 93:1168–1180.

Kingsley DM (1994) The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. Genes and Development 8(2): 133-146

Kinnamon JC, Henzler DM, Royer SM (1993) HVEM ultrastructural analysis of mouse fungiform taste buds, cell types, and associated synapses. Microscopy Research and Technique 26: 142–156

Kolbeck R, Jungbluth S, Barde YA (1994) Characterisation of neurotrophins dimmers and monomers. European Journal of Biochemistry 225 (3): 995-1003

Korte M, Carroll P, Wolf E, Brem G, Thoenen H, Bonhoeffer T (1995) Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. Proceedings of the National Academy of Sciences of the USA 92(19): 8856-60

Krimm RF, Hill DL (1998) Innervation of single fungiform taste buds during development in rat. Journal of Comparative Neurology 398:13-24

Krimm RF, Miller KK, Kitzman PH, Davis MD, Albers KM (2001) Epithelial overexpression of BDNF or NT4 disrupts targeting of taste neurons that innervate the anterior tongue. Developmental Biology. 232: 508-521

Krimm RF (2006) Mice lacking the p75 receptor fail to acquire a normal complement of taste buds and geniculate ganglion neurons by adulthood. The anatomical record, Part A: Discoveries in molecular, cellular and evolutionary biology. 288(12): 1294-302

Krimm RF (2007) Factors that regulate embryonic gustatory development. BMC Neuroscience 8: Suppl 3:S4

Krimm RF, Barlow LA (2008) Development of the taste system. The Senses: A Comprehensive Reference. 4: 157-187

Lee KF, Bachman K, Landis S, Jaenisch R (1994) Dependence on p75 for innervation of some sympathetic targets. Science 263:1447–49

Lee R, Kermani P, Teng KK, Hempstead BL (2001a) Regulation of cell survival by secreted proneurotrophins. Science 294:1945–1948.

Lee FS, Kim AH, Khursigara G, Chao MV (2001b) The uniqueness of being a neurotrophin receptor. Current Opinion in Neurobiology 11(3):281-6

Lee FS, Rajagopal R, Kim AH, Chang PC, Chao MV (2002) Activation of Trk neurotrophins receptor signalling by pituitary adenylate cyclise-activating polypeptides. Journal of Biological Chemistry 277(11)9096-102

LeMaster AM, Krimm RF, Davis BM, Noel T, Forbes ME, Johnson JE, Albers KM (1999) Overexpression of brain-derived neurotrophic factor enhances sensory innervation and selectively increases neuron number. Journal of Neuroscience 19: 5919-5931

Lendahl U, Zimmerman LB, McKay RD (1990) CNS stem cells express a new class of intermediate filament protein. Cell 60: 585-595

Lessmann V, Gottmann K, Malcangio M (2003) Neurotrophin secretion: current facts and future prospects. Progress in Neurobiology 69 (5): 341-74

Li X, Staszewski L, Xu H, Durick K, Zoller M, Adler E (2002) Human receptors for sweet and umami taste. Proceedings of the National Academy of Sciences of the USA 99(7): 4692-6

Liebl DJ, Tessarollo L, Palko ME, Parada LF (1997) Absence of sensory neurons before target innervation in Brain-derived neurotrophic factor-, Neurotrophin 3-, and TrkC-deficient embryonic mice. The Journal of Neuroscience. 17(23):9113-9121

Liebl DJ, Mbiene J-P, Parada LF (1999) NT4/5 mutant mice have deficiency in gustatory papillae and taste bud formation. Developmental Biology. 213: 378-389

Lindemann B (2001). Receptors and transduction in taste. Nature 413: 219–225

Liu X, Ernfors P, Wu H, Jaenisch R (1995) Sensory but not motor neuron deficits in mice lacking BDNF and NT4. Nature 375: 238-241

Liu X, Jaenisch R (2000) Severe peripheral sensory neuron loss and modest motor neuron reduction in mice with combined deficiency of brain-derived neurotrophic factor, neurotrophin 3 and neurotrophin 4/5. Developmental Dynamics 218:94–101

Liu HX, Maccallum DK, Edwards C, Gaffield W, Mistretta CM (2004) Sonic hedgehog exerts distinct, stage-specific effects on tongue and taste papilla development. Developmental Biology 276:280-300.

Liu F, Thirumangalathu S, Gallant NM, Yang SH, Stoick-Cooper CL, Reddy ST, Andl T, Taketo MM, Dlugosz AA, Moon RT (2007) Wnt-beta-catenin signaling initiates taste papilla development. Nature genetics 39:106-112.

Liu H, Henson BS, Zhou Y, D'Silva NJ, Mistretta CM (2008) Fungiform papilla pattern: EGF regulates inter-papilla lingual epithelium and decreases papilla number via PI3K/Akt, Mek/Erk and p38 MAPK signalling. Developmental Dynamics 237(9):2378-2393

Liu HX, Grosse AS, Iwatsuki K, Mishina Y, Gumucio DL, Mistretta CM (2012) Separate and distinctive roles for Wnt5a in tongue, lingual tissue and taste papilla development. Developmental Biology 361(1): 39-56

Lisman J, Schulman H, Cline H (2002) The molecular basis of CaMKII function in synaptic and behavioural memory. Nature Reviews Neuroscience 3: 175–190

Lonze BE, Riccio A, Cohen S, Ginty DD (2002) Apoptosis, axonal growth defects, and degeneration of peripheral neurons in mice lacking CREB. Neuron 34(3):371-85

Lopez GF, Krimm RF (2006) Refinement of innervation accuracy following initial targeting of peripheral gustatory fibers. Journal of Neurobiology 66(10): 1033-1043

Lu, B (2003) Pro-region of neurotrophins: role in synaptic modulation. Neuron 39, 735–738

Lu B, Pang PT, Woo NH, The yin and yang of neurotrophins action (2005) Nature Reviews Neuroscience 6(8): 603-14

Ma Q, Chen Z, del Barco Barrantes I, de la Pompa JL, Anderson DJ (1998) Neurogenin1 is essential for the determination of neuronal precursors for proximal cranial sensory ganglia. Neuron 20:469-482

Ma L, Lopez GF, Krimm RF (2009) Epithelial-derived brain-derived neurotrophic factor is required for gustatory neuron targeting during a critical developmental period. The Journal of Neuroscience 29(11): 3354-3364

Maggirwar SB, Sarmiere PD, Dewhurst S, Freeman RS (1998) Nerve growth factor-dependent activation of NF-kappaB contributes to survival of sympathetic neurons. Journal of Neuroscience 18:10356–10365.

Mangold JE, Hill DL (2008) Postnatal reorganization of primary afferent terminal fields in the rat gustatory brainstem is determined by prenatal dietary history. Journal of Comparative Neurology 509(6):594-607

Marler KJ, Becker-Barosso E, Martinez A, Llovera M, Wentzel C, Poopalasundaram S, Hindges R, Soriano E, Comella J, Drescher U (2008) A TrkB/EphrinA interaction controls retinal axon branching and synaptogenesis. The Journal of Neuroscience 28(48):12700-12

May OL, Hill DL (2006) Gustatory terminal field organization and developmental plasticity in the nucleus of the solitary tract revealed through triple-fluorescence labelling. Journal of Comparative Neurology 497(4): 658-69

Mbiene JP, Mistretta CM (1997) Initial innervation of embryonic rat tongue and developing taste papillae: nerves follow distinctive and spatially restricted pathways. Acta Anatomica (Basel) 160:139-158.

Mbiene JP, Roberts JD (2003) Distribution of keratin 8-containing cell clusters in mouse embryonic tongue: evidence for a prepattern for taste bud development. Journal of Comparative Neurology 457(2):111-22

Mbiene JP (2004) Taste placodes are primary targets of geniculate but not trigeminal sensory axons in mouse developing tongue. Journal of Neurocytology 33:617-629

Meakin SO, MacDonald JI, Gryz EA, Kubu CJ, Verdi HM (1999) The signaling adapter FRS-2 competes with Shc for binding to the nerve growth factor receptor TrkA. A model for discriminating proliferation and differentiation. Journal of Biological Chemistry 274(14):9861-70

Medina DL, Sciarretta CS, Calella AM, von Bohlen und Halbach O, Unsicker K, Minichiello L (2004) TrkB regulates neocortex formation through the Shc/PLCγ-mediated control of neuronal migration. The EMBO Journal 23: 3803-3814

Minichiello L, Klein R (1996) TrkB and TrkC neurotrophin receptors cooperate in promoting survival of hippocampal and cerebellar granule neurons. Genes and Development 10:2849–2858.

Minichiello L, Casagranda F, Tatche RS, Stucky CL, Postigo A, Lewin GR Davies AM, Klein R (1998) Point mutation in trkB causes loss of NT4-dependent neurons without major effects on diverse BDNF responses. Neuron: 21,335-345

Minichiello L, Calella AM, Medina DL, Bonhoeffer T, Klein R, Korte M (2002) Mechanism of TrkB-mediated hippocampal long-term potentiation. Neuron 36: 121-137

Minichiello L (2009) TrkB signalling pathways in LTP and learning. Nature Reviews Neuroscience 10 (12): 850-860

Mistretta CM, Goosens KA, Farinas I, Reichardt LF (1997) Alterations in size, number and morphology of gustatory papillae and taste buds in BDNF null mutant mice demonstrate neural dependence of developing taste organs. The Journal of Comparative Neurology 409: 13-24

Mistretta CM (1998) The role of innervation in induction and differentiation of taste organs: introduction and background. Annals of the New York Academy of Sciences 855: 1-13

Mistretta CM, Goosens KA, Farinas I, Reichardt LF (1999) Alterations in size, number, and morphology of gustatory papillae and taste buds in BDNF null mutant mice demonstrate neural dependence of developing taste organs. Journal of Comparative Neurology 409:13-24.

Mistretta CM, Liu HX, Gaffield W, MacCallum DK (2003) Cyclopamine and jervine in embryonic rat tongue cultures demonstrate a role for Shh signaling in taste papilla development and patterning: fungiform papillae double in number and form in novel locations in dorsal lingual epithelium. Developmental Biology 254:1-18

Mistretta CM, Liu HX (2006) Development of fungiform papillae: patterned lingual gustatory organs. Archives of Histology and Cytology 69:199-208.

Nagato T, Matsumoto K, Tanioka H, Kodama J, Toh H (1995): Effect of denervation on morphogenesis of the rat fungiform papilla. Acta Anatomica (Basel)153:301-309

Naumann T, Casademunt E, Hollerbach E, Hofmann J, Dechant G, Frotscher M, Barde YA (2002) Complete deletion of the neurotrophin receptor p75NTR leads to long-lasting increases in the number of basal forebrain cholinergic neurons. The Journal of Neuroscience 22:2409-2418.

Nelson G, Hoon MA, Chandrashekar J, Zhang Y, Ryba NJ, Zuker CS (2001) Mammalian sweet taste receptors. Cell 106:381–390

Nelson G, Chandrashekar J, Hoon MA, Feng L, Zhao G, Ryba NJ, Zuker CS (2002) An amino-acid taste receptor. Nature. 416:199–202

Nosrat CA, Ebendal T, Olson L (1996) Differential expression of brain-derived neurotrophic factor and neurotrophin 3 mRNA in lingual papillae and taste buds indicates roles in gustatoryand somatosensory innervation. Journal of Comparative Neurology 376:587-602

Nosrat CA, Blomlof J, ElShamy WM, Ernfors P, Olson L (1997) Lingual deficits in BDNF and NT3 mutant mice leading to gustatory and somatosensory disturbances, respectively. Development 124:1333-1342.

Nosrat CA, MacCallum DK, Mistretta CM (2001) Distinctive spatiotemporal expression patterns for neurotrophins develop in gustatory papillae and lingual tissues in embryonic tongue organ cultures. Cell Tissue Research 303:35-45.

Nosrat IV, Agerman K, Marinescu A, Ernfors P, Nosrat CA (2004) Lingual deficits in neurotrophins double knockout mice. Journal of Neurocytology 33: 607-615

Nykjaer A, Lee R, Teng KK, Jansen P, Madsen P, Nielsen MS, Jacobsen C, Kliemannel, Schwarz E, Willnow TE, Hempstead BL, Petersen CM (2004) Sortilin is essential for proNGF-induced neuronal cell death. Nature 427:843–848.

Oakley B, Labelle DE, Riley RA, Wilson K, Li LW (1991) The rate and locus of development of rat vallate taste buds. Developmental Brain Research 58: 215-221

Oakley B (1998) Taste neurons have multiple inductive roles in mammalian gustatory development. Annals of the New York Academy of Sciences 855: 50-57

Oakley B, Witt M (2004) Building sensory receptors on the tongue. Journal of Neurocytology 33:631-646

Oka Y, Butnaru M, von Buchholtz L, Ryba NJP, Zuker CS (2013) High salt recruits aversive taste pathways. Nature 494: 472-475

Okubo T, Pevny LH, Hogan BL (2006) Sox2 is required for development of taste bud sensory cells. Genes and Development 20:2654-2659.

Okubo T, Clark C, Hogan BL (2008) Cell lineage mapping of taste bud cells and keratinocytes in the mouse tongue and soft palate. Stem Cells 27(2): 442-50

Patel AV, Krimm RF (2010a) BDNF is required for the survival of differentiated geniculate ganglion neurons. Developmental Biology 340: 419-429

Patel AV, Huang T, Krimm RF (2010b) Lingual and palatal gustatory afferents each depend on both BDNF and NT-4, but the dependence is greater for lingual than palatal afferents. Journal of Comparative Neurology 518:3290-3301

Patel AV, Krimm RF (2012) Neurotrophin-4 regulates the survival of gustatory neurons earlier in development using a different mechanism than brain-derived neurotrophic factor. Developmental biology

Patterson SL, Pittenger C, Morozov A, Martin KC, Scanlin H, Drake CT, Kandel ER (2001) Some forms of cAMP-mediated long-lasting potentiation are associated with release of BDNF and nuclear translocation of phospho-MAP kinase. Neuron 32:123– 140.

Perez P, Coll PM, Hempstead BL, Martin-Zanca D, Chao MV (1995) NGF binding to the trk tyrosine kinase receptor requires the extracellular immunoglobulin-like domains. Molecular and Cellular Neurosciences 6, 97–105.

Qian D, Radde-Gallwitz K, Kelly M, Tyrberg B, Kim J, Gao W, Chen P (2006) Basic helix-loop-helix gene Hes6 delineates the sensory hair cell lineage in the inner ear. Developmental Dynamics 235:1689-1700

Qian X, Riccio A, Zhang Y, Ginty DD (1998) Identification and characterization of novel substrates of Trk receptors in developing neurons. Neuron 21:1017-1029

Qian X, Ginty DD (2001) SH2-B and APS are multimeric adapters that augment TrkA signalling. Molecular Cell Biology 21(5):1613-20

Rajagopal R, Chao MV (2006) A role for Fyn in Trk receptor transactivation by G-protein coupled receptor signaling. Molecular Cell Neuroscience 33:36–46.

Rattenholl, A Ruoppolo M, Flagiello A, Monti M, Vinci F, Marino g, Lilie H, Schwarz E, Rudolph (2001) Pro-sequence assisted folding and disulfide bond formation of human nerve growth factor. Journal of Molecular Biology 305, 523–533

Reichardt LF (2006) Neurotrophin-regulated signalling pathways. Philosophical Transactions of the Royal Society of London. Series B Biological Sciences 361:1545–1564

Riccio A, Ahn S, Davenport CM, Blendy JA, Ginty DD (1999) Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons. Science 286:2358–2361.

Ringstedt T, Ibanez CF, Nosrat Ca (1999) Role of brain-derived neurotrophic factor in target invasion in the gustatory system. The Journal of Neuroscience 19 (9): 3507-3518

Rochlin MW, Farbman AI (1998) Trigeminal ganglion axons are repelled by their presumptive targets. Journal of Neuroscience 18(17): 6840-52

Rochlin MW, O'Connor R, Giger RJ, Verhaagen J, Farbman AI (2000) Comparison of neurotrophin and repellent sensitivities of early embryonic geniculate and trigeminal axons. Journal of Comparative Neurology 422:579-593.

Rui L, Herrington J, Carter-Su C (1999) SH2-B is required for nerve growth factor-induced neuronal differentiation. Journal of Biological Chemistry 274(15):10590-4

Runge EM, Hoshino N, Biehl MJ, Ton S, Rochlin MW (2012) Neurotrophin-4 is more potent than brain-derived neurotrophic factor in promoting, attracting and suppressing geniculate ganglion neurite outgrowth. Developmental Neuroscience 2-13

Saito N, Shirai Y (2002) Protein kinase C gamma (PKC gamma): function of neuron specific isotype. Journal of Biochemistry 132:683–687

Schecterson LC, Bothwell M (2010) Neurotrophin receptors: Old friends with new partners. Developmental Neurobiology 70:332–338.

Schlosser G (2006) Induction and specification of cranial placodes. Developmental Biology 294: 303-351

Seta Y, Seta C, Barlow LA (2003) Notch-associated gene expression in embryonic and adult taste papillae and taste buds suggests a role in taste cell lineage decisions. Journal of Comparative Neurology 464:49-61.

Sciarretta C, Fritzsch B, Beisel K, Rocha-Sanchez SM, Buniell A, Horn JM, Minichiello L (2010) PLCγ-activated signaling is essential of TrkB mediated sensory neuron structural plasticity. BMC Developmental Biology 10:103

Shi Y, MantuanoE, Inoue G, Campana WM, Gonias SL (2009) Ligand binding to LRP1 transactivates Trk receptors by a Src family kinase-dependent pathway. Science Signaling 2(68):ra18

Smeyne RJ, Klein R, Schnapp A, Long LK, Bryant S, Lewin A, Lira SA, Barbacid M (1994) Severe sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene. Nature 368:246–249

Smith D V, St. John SJ (1999) Neural coding of gustatory information. Current Opinion in Neurobiology 9: 427–435

Soderling TR (2000) CaM-kinases: modulators of synaptic plasticity. Current Opinion in Neurobiology 10:375–380.

Sollars SI, Smith PC, Hill DL (2002) Time course of morphological alterations of fungiform papillae and taste buds following chorda tympani transaction in neonatal rats. Journal of Neurobiology 51(3)223-36

Sollars SI (2005) Chorda tympani nerve transaction at different developmental ages produces differential effects on taste bud volume and papillae morphology in the rat. Journal of Neurobiology 64(3): 310-20

Sollars SI, Hill DL (2005) In vivo recordings from rat geniculate ganglia: taste response properties of individual great superficial petrosal and chorda tympani neurons. Journal of Physilogy 564: 877-93

Sollars SI, Walker BR, Thaw AK, Hill DL (2006) Age-related decrease of the chorda tympani nerve terminal field in the nucleus of the solitary tract is prevented by dietary sodium restriction during development. Neuroscience 137(4): 1229-36

St John SJ, Markison S, Spector AC (1995) Salt discriminability is related to number of regenerated taste buds after chorda tympani nerve section in rats. The American Journal of Physiology 269: R141-53

Stone LM, Tan SS, Tam PP, Finger TE (2002) Analysis of cell lineage relationships in taste buds. Journal of Neuroscience 22(11): 4522-9

Strohmaier C, Carter B, Urfer R, Barde YA, Dechant G (1996) A splice variant of the neurotrophin receptor TrkB with increased specificity for brain-derived neurotrophic factor. EMBO Journal. 15:3332–37

Sun W, Kesavan K, Schaefer BC, Garrington TP, Ware M, Johnson NL, Gelfand EW, Johnson GL (2001) MEKK2 associates with the adapter protein Lad/RIBP and regulates MEK-BMK1/ERK pathway. Journal of Biological Chemistry 276(7):5093-100

Sun H and Oakley B (2002) Development of anterior gustatory epithelia in the palate and tongue requires epidermal growth factor receptor. Developmental Biology 242: 31–43

Suter, U., Heymach, J. V. Jr & Shooter, E. M. (1991) Two conserved domains in the NGF propeptide are necessary and sufficient for the biosynthesis of correctly processed and biologically active NGF. EMBO Journal. **10**, 2395–2400

Sweatt JD (1999) Toward a molecular explanation for long-term potentiation. Learning & Memory 6: 399-416

Teng HK, Teng KK, Lee R, Wright S, Tevar S, Almeida RD, Kermani P, Torkin R, Chen Z-Y, Lee FS, Kraemer RT, Nykjaer A, Hempstead BL (2005) ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin. The Journal of Neuroscience 25:5455–5463.

Tessarollo L, Tsoulfas P, Donovan MJ, Palko MR, Blair-Flynn J, Hempstead BL, Parada LF (1997). Targeted deletion of all isoforms of the *trkC* gene suggests the use of alternate receptors by its ligand neurotrophin-3 in neuronal development and implicates *trkC* in normal cardiogenesis. Proceedings of the National Academy of Sciences of the USA. 94(26):14776-14781

Thirumangalathu S, Harlow DE, Driskell AL, Krimm RF, Barlow LA (2009) Fate mapping of mammalian embryonic taste bud progenitors. Development 136 (9): 1519-1528

Toh H, Rittman G, Mackenzie IC (1993) Keratin expression in taste bud cells of the circumvallate and foliate papillae of adult mice. Epithelial Cell Biology 2(3): 126-33

Tomchik SM, Berg S, Kim JW, Chaudhari N, Roper SD (2007)Breadth of tuning and taste coding in mammalian taste buds. Journal of Neuroscience 27:10840–10848

Tusa RJ, Palmer LA, Rosenquist AC (1978) The retinotopic organization of area 17 (striate cortex) in the cat. Journal of Comparative Neurology 177(2): 213-35

Urfer R, Tsoulfas P, O'Connell L, Shelton DL, Parada LF, Presta LG (1995) An immunoglobulin-like domain determines the specificity of neurotrophin receptors EMBO Journal 14:2795–2805.

Vandenbeuch A, Clapp TR, Kinnamon SC (2008) Amiloride-sensitive channels in type I fungiform taste cells in mouse. BMC Neuroscience 9:1

Vandenbeuch A, Kinnamon SC (2009) Why do taste cells generate action potentials? Journal of Biology 8(4): 42

Vilar M, Charalampopoulos I, Kenchappa RS, Reversi A, Klos-Applequist JM, Karaca E, Simi A, Spuch C, Choi S, Friedman WJ, Ericson J, Schiavo G, Carter BD, Ibanez CF (2009a) Ligand-independent signaling by disulfide-crosslinked dimers of the p75 neurotrophin receptor. The Journal of Cell Science 122:3351–3357.

Vilar M, Charalampopoulos I, Kenchappa RS, Simi A, Karaca E, Reversi A, Choi S, Bothwell M, Mingarro I, Friedman WJ, Schiavo G, Bastiaens PIH, Verveer PJ, Carter BD, Ibanez CF (2009b) Activation of the p75 neurotrophin receptor through conformational rearrangement of disulphide-linked receptor dimers. Neuron 62:72–83

Vilbig R, Cosmano J, Giger R, Rochlin MW (2004) Distinct roles for Sema3A, Sema3Fm and an unidentified trophic factor in controlling the advance of geniculate axons to gustatory lingual epithelium. Journal of Neurocytology 33(6):591-606

Whitehead MC, Kachele DL (1994) Development of fungiform papillae, taste buds, and their innervation in the hamster. Journal of Comparative Neurology 340(4): 515-30

Witt M, Reutter K (1998) Innervation of developing human taste buds. Histochemistry and Cell Biology 109(3): 281-91

Xian CJ, Zhou EF (2000) Roles of transforming growth factor-alpha and related molecules in the nervous system. Molecular Neurobiology 20: 157–183

Xing J, Kornhauser JM, Xia Z, Thiele EA, Greenberg ME (1998) Nerve growth factor activates extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways to stimulate CREB serine 133 phosphorylation. Molecular Cell Biology 18:1946–1955.

Yamada M, Ohnishi H, Sano S i, Nakatani A, Ikeuchi T, Hatanaka H (1997) Insulin receptor substrate (IRS)-1 and IRS-2 are tyrosine-phosphorylated and associated with phosphatidylinositol 3-kinase in response to brain-derived neurotrophic factor in cultured cerebral cortical neurons. Journal of Biological Chemistry 272:30334–30339.

Yamout A, Spec A, Cosmano J, Kashyap M, Rochlin MW (2005) Neurotrophic factor receptor expression and in vitro nerve growth of geniculate ganglion neurons that supply divergent nerves. Developmental Neuroscience 27: 288-298

Yarmolinsky DA, Zuker CS, Ryba NJP (2009) Common sense about taste: from mammals to insects. Cell 139: 234-244

Ye H, Kuruvilla R, Zweifel LS, Ginty DD (2003) Evidence in support of signalling endosome-based retrograde survival of sympathetic neurons. Neuron 39:57–68

Yee CL, Jones KR, Finger TE (2003) Brain-derived neurotrophic factor is present in adult mouse taste cells with synapses. Journal of Comparative Neurology 459: 15-24

Yuan XB, Jin M, Xu X, Song YQ, Wu CP, Poo MM, Duan S (2003) Signalling and crosstalk of Rho GTPases in mediating axon guidance. Nat Cell Biol 5:38–45.

Zaccaro MC, Ivanisevic L, Perez P, Meakin SO, Saragovi HU (2001) p75 Co-receptors regulate ligand-dependent and ligand-independent Trk receptor activation, in part by altering Trk docking subdomains. Journal of Biological Chemistry 276(33):31023-9

Zaidi FN, Whitehead MC (2006) Discrete innervation of murine taste buds by peripheral taste beurons. Journal of Neuroscience 26(32): 8243-53

Zhao GQ, Zhang Y, Hoon MA, Chandrashekar J, Erlenbach I, Ryba NJ, Zuker CS (2003) The receptors for mammalian sweet and umami taste Cell 115:255–266

Zhang C, Brandemihl A, Lau D, Lawton A, Oakley B (1997) BDNF is required for the normal development of taste neurons in vivo. Neuroreport 8:1013-1017.

Zhou Y, Liu HX, Mistretta CM (2006) Bone morphogenetic proteins and noggin: inhibiting and inducing fungiform taste papilla development. Developmental Biology 297:198-213.

Zou D, Silvius D, Fritzsch B, Xu PX (2004) Eya1 and Six1 are essential for early steps of sensory neurogenesis in mammalian cranial placodes. Development 2004, 131:5561-5572.